Studies on Pseudomonas aeruginosa citrate synthase.

Solomon, M.

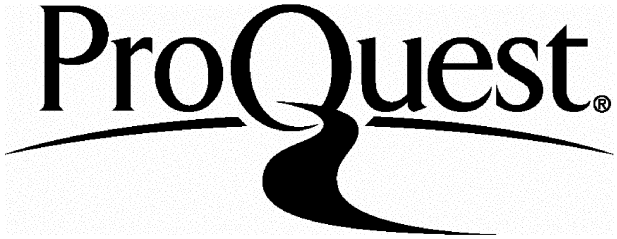
Award date:
1981

Awarding institution:
University of Bath

STUDIES ON PSEUDOMONAS AERUGINOSA CITRATE SYNTHASE

Submitted by Michael Solomon, B.Sc.
for the degree of Ph.D. of the
University of Bath
1981

M. Solomon



To my parents

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisor, Professor P.D.J. Weitzman, for his encouragement and guidance through this work.

I wish to thank Mrs G. Asbridge and Miss H. Kinghorn for their technical assistance and the Science Research Council for the award of a Research Studentship.

Finally, I would like to express my gratitude to Mrs J. Williams for typing this thesis.

ABBREVIATIONS

Most of the abbreviations used in this thesis are those recommended in the Biochemical Society publication "Policy of the Journal and Instructions to Authors". (Biochem.J. (1978) 169, 1-27)

Non-standard abbreviations:

EDC : 1 ethyl-3-(3-dimethylaminopropyl) carbodiimide

DTNB : 5,5'-dithiobis-(2-nitrobenzoic acid)

DTNP : 2,2'-dithiobis-(5-nitropyridine)

NTCB : 2-nitro-5-thiocyanobenzoic acid

2-PDS : 2,2'-dipyridyl disulphide

4-PDS : 4,4'-dipyridyl disulphide

DTT : dithiothreitol

'Tris buffer (pH 8.0)': 20 mM-Tris-HCl (pH 8.0), 1 mM-EDTA

'Tris buffer (pH 7.0)': 20 mM-Tris-HCl (pH 7.0), 1 mM-EDTA

SUMMARY

Two distinct citrate synthases were found in a mutant Pseudomonas aeruginosa. A 'large' enzyme (CS I; mol.wt.≈300,000), allosterically inhibited by NADH and activated by AMP, predominates in a log-phase (nutrient-glutamate) culture whereas a 'small' (non-regulated) enzyme (CS II; mol.wt.≈100,000) predominates in stationary phase. By contrast, wild-type Ps.aeruginosa contains only one citrate synthase - a 'large' enzyme, allosterically inhibited by NADH and deinhibited by AMP. This growth-stage dependent enzyme variation in the mutant represents a quite novel finding.

Growth on different carbon sources revealed considerable variation in the ratio of the two mutant enzymes. It appears that CS II is not a breakdown product of CS I. Treatment of a growing nutrient-glutamate culture with chloramphenicol resulted in no change in the ratio of the two enzymes during stationary phase; indicating the probability of de novo synthesis of CS II.

Matrex Gel Red A, a group-specific ligand, was used in the purification of the Pseudomonas citrate synthases and, together with other conventional procedures, resulted in considerable purification of all three enzymes. Several affinity ligands were prepared using substrates and effectors of citrate synthase but none was useful in the purification of the Pseudomonas enzymes.

The catalytic and regulatory properties of the wild-type and mutant enzymes were compared. Photo-oxidation with Methylene Blue and treatment with the thiol-blocking reagent 5,5'-dithiobis-(2-nitrobenzoic acid) both resulted in a rapid loss of sensitivity to inhibition by NADH and a slower loss of activation by AMP, indicating that the enzyme contains separate NADH and AMP binding sites. A similar situation may occur in the wild-type enzyme.

Considerable variation was found in both the sensitivity of diverse citrate synthases to various thiol-blocking reagents and the effect of salt on the reactivity of these reagents.

CONTENTS

INTRODUCTION

The Function of the Citric Acid (CA) Cycle

The citric acid (CA) cycle was first proposed in 1937 by Krebs and Johnson to account for the terminal stages of carbohydrate oxidation in pigeon breast muscle. It has subsequently been shown that this metabolic cycle operates for the final oxidation of all major foodstuffs in respiring organisms (Kornberg, 1959; Krebs and Lowenstein, 1960).

Metabolic pathways have two major functions: one is the catabolism (or degradation) of compounds to yield energy and the other is the anabolism (or biosynthesis) of cell components. The term amphibolic was proposed by Davis (1961) to describe a pathway which fulfils both an anabolic and a catabolic function. It can be seen from Fig.1 that the CA cycle fulfils both of these functions.

The catabolic role of the CA cycle is achieved by the oxidation of acetyl moieties to carbon dioxide and reduced pyridine nucleotides; the latter may be re-oxidised via the electron transport chain with the production of ATP as an energy source.

The anabolic role of the CA cycle was proposed by Krebs *et al*. (1952) who provided isotopic evidence that

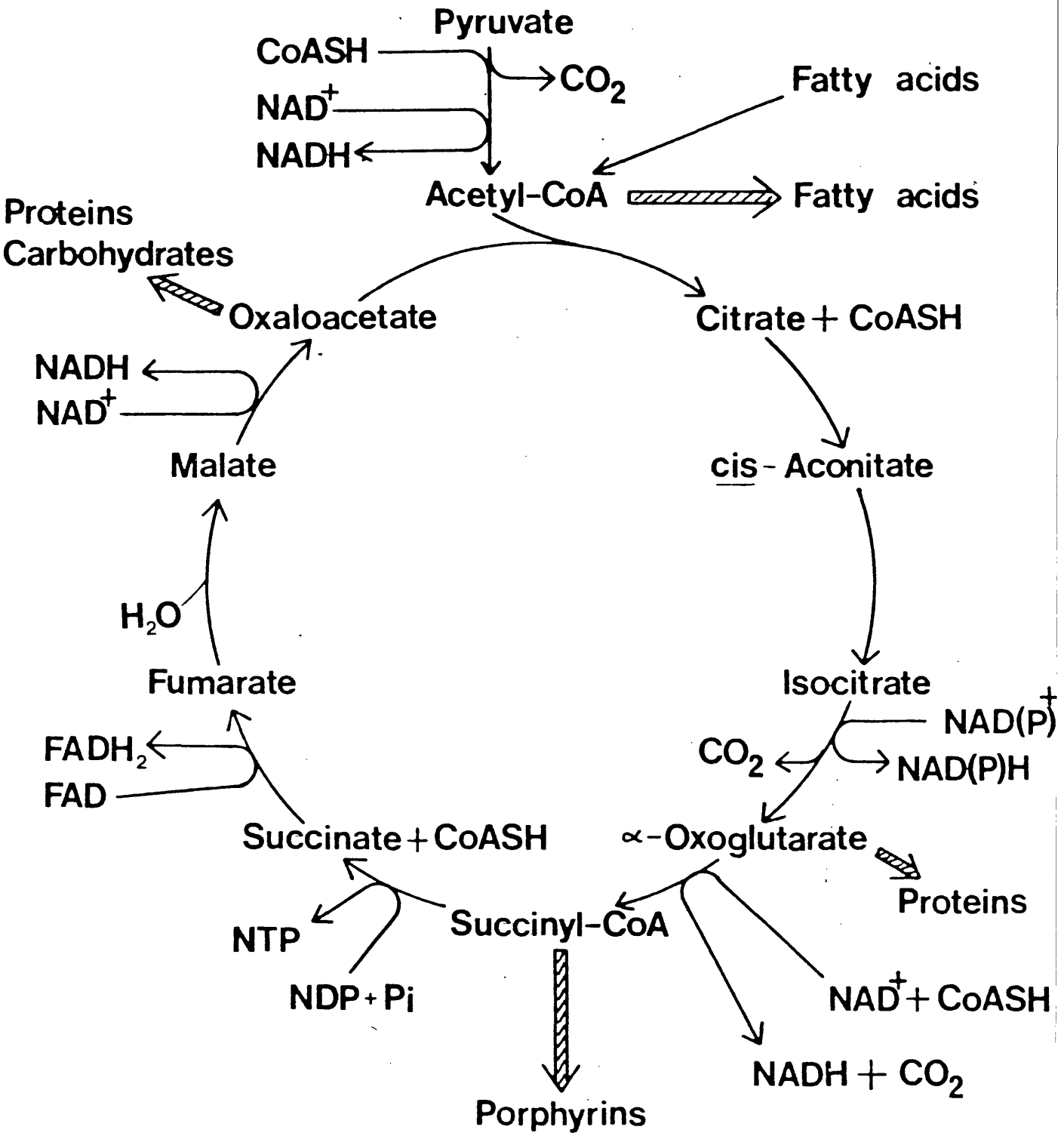


Fig.1. The role of the citric acid cycle in the production of energy and cell constituents

the cycle served to supply intermediates for synthetic processes in Escherichia coli. Roberts et al. (1953) showed that amino acids were derived from metabolites of the CA cycle.

A complete functional CA cycle is found, almost without exception, in all aerobic and facultatively anaerobic chemo-organotrophs. However, when facultative anaerobes (e.g. E.coli) are grown anaerobically they lose the ability to derive ATP from reduced pyridine nucleotides and consequently the catabolic role of the cycle is lost. Amarasingham and Davis (1965) showed that under these conditions α-oxoglutarate dehydrogenase was absent but that the anabolic functions were maintained by a non-cyclic pathway (Fig.2).

The Discovery of Citrate Synthase

Stern and Ochoa (1949) prepared an ammonium sulphate extract from pigeon liver which catalysed the formation of citrate from acetate, ATP, CoASH and oxaloacetate. Stern et al. (1950) separated this activity into two enzyme components, one catalysing the formation of 'active' acetate and the other, "condensing enzyme", which could effect the combination of 'active' acetate with oxaloacetate to produce citrate. 'Active' acetate was

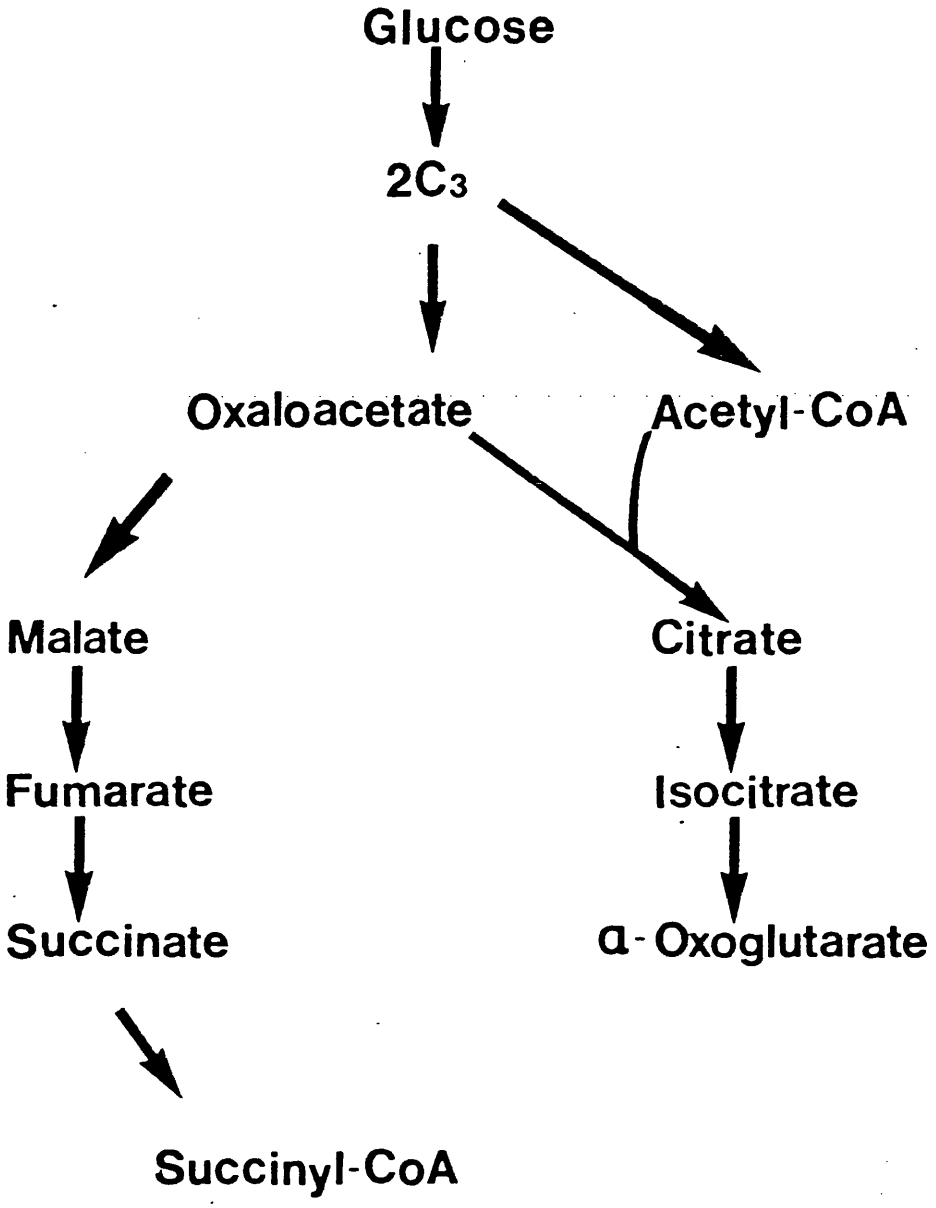


Fig.2. Modified citric acid cycle in E.coli
Scheme proposed by Amarasingham and Davis (1965) for E.coli growing under anaerobic conditions.

later identified as acetyl-CoA (Lynen and Reichert, 1951).

"Condensing enzyme" was subsequently purified from pig heart and was the first enzyme of the CA cycle to be obtained in crystalline form (Ochoa et al.,1951). "Condensing enzyme", now known as citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7), catalyses the following reaction:

$$\text{Oxaloacetate}^{2-} + \text{acetyl-CoA} + H_2O = \text{citrate}^{3-} + \text{CoASH} + H^+$$

The enzyme has been found in animal tissues, bacteria and yeasts (Stern and Ochoa, 1951), fungi (Ramakrishnan and Martin, 1954) and plants (Deshpande and Ramakrishnan, 1959),and has more recently been investigated from a variety of sources, e.g. pig heart (Bayer et al.,1981 a, b), E.coli and Acinetobacter (Danson and Weitzman, 1973, 1977; Morse and Duckworth, 1980) and Neurospora crassa (Harmey and Neupert, 1979). Instances of its apparent absence have been reported in Lactobacillus plantarum, Streptococcus faecalis and Gemella haemolysans (Weitzman and Danson, 1976).

Citrate synthase may be considered the 'initial' enzyme of the cycle insofar as it effects the entry of carbon, in

the form of acetyl units, into the cycle, each turn of which leads to the total oxidation to carbon dioxide of an equivalent amount of carbon. Furthermore, citrate synthase is the only enzyme of the cycle which catalyses the formation of a carbon-carbon bond.

Citrate Synthase and the Regulation of the CA Cycle

The central role of the CA cycle in the metabolism of the cell necessitates its strict control. As citrate synthase can be regarded as the 'initial' enzyme of the cycle one would expect it to contribute towards the overall control of the cycle.

The observations that cycle intermediates did not accumulate and that addition of such intermediates produced an increased rate of oxidation led Krebs and Lowenstein (1960) to conclude that the rate-limiting step of the cycle was the synthesis of citrate and that primary control of the cycle must be exerted on citrate synthase, the enzyme responsible for the synthesis of citrate. Even though other enzymes of the cycle may be regulated, citrate synthase is responsible for the introduction of carbon and therefore the control of the enzyme will regulate the flux of carbon through the cycle. It is therefore not surprising that the molecular, catalytic

and regulatory properties of citrate synthase have been the subjects of intensive investigation. Several reviews of the field have been published (Srere, 1971; Spector, 1972; Srere, 1972; Weitzman and Danson, 1976; Weitzman, 1981).

Control Mechanisms of Citrate Synthase

The rate at which the ultimate products of a metabolic pathway are formed can only be controlled by changing the activity of the rate-limiting enzyme in that pathway. Citrate synthase being regarded as the 'initial' enzyme of the CA cycle must be a strong candidate for this role. Enzyme rate is a function of pH and intracellular concentrations of substrates, products and cofactors. Dixon and Webb (1964) stated that the concentrations of substrates approached the Km values for their enzymes and therefore any alteration in the availability of acetyl-CoA or oxaloacetate, the substrates of citrate synthase, may play an important role in the control of this enzyme. The regulation of citrate synthase (and any other regulatory enzyme) can be divided into two components.

1. Genetic control of enzyme synthesis

This is a 'coarse' or 'long term' control of enzyme activity exerted by regulating the total amount of enzyme

present. The rate of a given metabolic sequence must depend on the concentration of active enzyme which is a result of the balance between the rates of its synthesis and degradation. In prokaryotes under normal growth conditions there is very little enzyme degradation or protein turnover (Koch and Levy, 1955; Larrabee et al., 1980); regulation of enzyme content is primarily determined by the rate of specific gene expression.

Enzymes present in constant amounts are constitutive whereas those enzymes synthesized in response to the presence of certain substrates or suitable derivatives are inducible. Catabolic pathways are subject to two types of genetic control - catabolic repression and substrate induction - whereas anabolic pathways are only subject to feedback repression of enzyme synthesis.

The levels of citrate synthase have been shown to vary depending on the nature of the carbon source. Gray et al. (1966) showed that citrate synthase (and all CA cycle enzymes) in facultative anaerobes are subject to catabolite repression (formerly known as the "glucose effect") which is a reduction in the rate of synthesis of certain enzymes in the presence of glucose (Magasanik, 1961). Polakis and Bartley (1965) showed that the levels of citrate synthase in yeast varied

when grown on different sugars.

The synthesis of β-galactosidase in E.coli was shown to be induced by the addition of lactose to the growth medium (Hogness et al., 1955). This was termed substrate induction of enzyme synthesis and it was shown by Umbarger (1954) that the synthesis of citrate synthase was induced by acetate in E.coli; the same metabolite also induced citrate synthase in Rhodopseudomonas capsulata (Eidels and Preiss, 1970).

In feedback repression the concentration of end product in the growth medium controls the rate of synthesis of the enzyme that is involved in the biosynthetic pathway of, for example, an amino acid. Thus Gorini and Maas (1957) showed that arginine inhibited the synthesis of ornithine transcarbamoylase. Citrate synthase in Bacillus subtilis has been found to be sensitive to feedback repression by glutamate (or α-oxoglutarate), the biosynthetic end product of the first half of the CA cycle (Flechtner and Hanson, 1969).

The CA cycle is an amphibolic pathway and therefore one would expect the cycle to be controlled by catabolic and anabolic signals. Separate and independent mechanisms

regulate the activity of the catabolic and anabolic reactions of the cycle (Sanwal, 1970). Once a regulatory enzyme is synthesized its activity is regulated by effectors ('fine' control).

2. Action of regulatory or allosteric enzymes

This can be regarded as a 'fine' or 'acute' control of enzyme activity. The concentration of active enzyme is frequently less than the total concentration of enzyme, by virtue of the binding of specific inhibitors, resulting in either a reduction of the proportion of the total number of enzyme molecules able to react with a fixed concentration of substrate, or an actual reduction in the catalytic efficiency of the enzyme. The process may be reversed by the simultaneous presence of activator ligands. The proportion of total active enzyme is a function of the relative concentrations of specific activators and inhibitors.

Many regulatory enzymes exhibit sigmoid rate response curves with increasing substrate concentration. The binding of effector ligands frequently alters the degree of such sigmoidicity, such that the effect of the ligand is markedly greater than that expected from enzymes which obey simple Michaelis-Menten kinetics.

The first step of an anabolic pathway is sensitive to feedback inhibition by the ultimate end product of that pathway. Thus Umbarger (1956) showed that threonine dehydratase, the first enzyme of the anabolic pathway for the formation of L-isoleucine and L-valine, is specifically inhibited by L-isoleucine, and Yates and Pardee (1956) showed that uracil or cytosine inhibit the formation of ureidosuccinic acid in the pyrimidine biosynthetic pathway of E.coli. Catabolic pathways are generally controlled by compounds which serve as indicators of the energy state of the cell. Histidine ammonia-lyase the initial enzyme of the histidine-degrading pathway in Pseudomonas aeruginosa is inhibited by inorganic pyrophosphate and activated by the low-energy signal AMP (Lessie and Neidhart, 1967) and phosphofructokinase in the mammalian glycolytic pathway is inhibited by the high-energy signal ATP and activated by the low-energy signals AMP, ADP and pyrophosphate (Passonneau and Lowry, 1962).

The regulation of citrate synthase has been found to be rather complex, its activity being sensitive to a number of different effectors.

(a) Adenine nucleotide inhibition

Hathaway and Atkinson (1965) discovered a direct inhibitory action of adenine nucleotides on the citrate synthase of yeast in cell-free extracts, and this inhibition was competitive with respect to acetyl-CoA. ATP produced the greatest effect, with ADP and AMP producing progressively weaker inhibitions. ATP can be regarded as the ultimate end product of the CA cycle and its effect can be regarded as an example of feedback inhibition. Atkinson (1968) suggested that the metabolic significance of these effects was dependent on the ratio of the three nucleotides rather than on their absolute effects ("energy charge" concept).

ATP inhibition has subsequently been demonstrated with citrate synthases from a variety of organisms including animals (Lee and Kosicki, 1967), bacteria (Flechtner and Hanson, 1969) and plants (Bogin and Wallace, 1966). Multiple-inhibition studies on various citrate synthases indicate that ATP probably acts as an isosteric inhibitor at the acetyl-CoA site (Harford and Weitzman, 1975).

(b) Specific NADH inhibition

The relative insensitivity of E.coli citrate synthase to ATP inhibition prompted a search for an alternative

regulator and led to the finding that NADH was a powerful and specific allosteric inhibitor of this enzyme competitive with acetyl-CoA (Weitzman, 1966 a, b). No inhibition was observed with NAD^+, $NADP^+$ or NADPH. Neither yeast nor pig heart citrate synthases were sensitive to NADH inhibition, whereas these two enzymes were far more sensitive to ATP inhibition than the E.coli enzyme. It was therefore suggested that in bacteria the role of ATP as a feedback regulator might be transferred to NADH.

Weitzman and Jones (1968) studied a number of bacteria and found that they fell into two distinct groups on the basis of the regulatory properties of their citrate synthases. Enzymes from Gram-negative bacteria were sensitive to NADH inhibition whereas no such inhibition was observed in Gram-positive bacteria. In addition the NADH-sensitive group of citrate synthases could be subdivided into two further groups on the basis of their response to AMP. The citrate synthases of strictly aerobic bacteria were found to have their NADH inhibition overcome by AMP whereas, with the enzymes from facultative anaerobes, AMP had no such effect. Weitzman and Jones (1968) suggested that the differences in the regulation of citrate synthase were due to the differences in the metabolic pathways of energy generation available to these

two classes of bacteria.

The facultative anaerobes use glycolysis as a major catabolic pathway and are able to generate energy by fermentation, using the CA cycle only for biosynthetic purposes. The strict aerobes depend totally on the CA cycle for energy production. The sensitivity of citrate synthase from strict aerobes to deinhibition by AMP may be in response to a low-energy signal thereby activating a key enzyme in the pathway responsible for energy production.

Citrate synthases from different organisms can also be divided into two distinct categories according to their molecular size. Weitzman and Dunmore (1969 a) showed that NADH-sensitive ('large') enzymes had molecular weights in the region of 250,000 whereas NADH-insensitive ('small') citrate synthases had molecular weights in the region of 100,000. This suggests that there is a correlation between a greater quaternary structure complexity and the response to NADH. In those cases where the subunit structure of the enzyme has been investigated the 'small' enzyme has been shown to be dimeric whereas the 'large' enzyme is probably hexameric.

A number of exceptions to the scheme outlined above have been reported, particularly within the Gram-negative group.

One of the more recently studied is the citrate synthase of Acetobacter xylinum, a 'large' enzyme which has been shown to be inhibited by ATP but is insensitive to NADH (Swissa and Benziman, 1976). Other exceptions are the citrate synthases from Halobacterium spp. and Thermus aquaticus which are of the 'small' type (Cazzulo, 1973; Weitzman, 1978). It is thought that a 'small' enzyme may be better able to maintain its native structure than a 'large' enzyme in the extreme conditions in which these latter organisms live. The halobacteria grow only in the presence of very high salt concentration (~4 M) and T.aquaticus has an optimum growth temperature of 70°C.

(c) α-Oxoglutarate inhibition

Wright et al. (1967) reported inhibition of E.coli citrate synthase by α-oxoglutarate, this inhibition being competitive with respect to oxaloacetate. Weitzman and Dunmore (1969 b) studied citrate synthases from a number of organisms and discovered that α-oxoglutarate inhibition occurred only with the Gram-negative facultative bacterial enzymes. These organisms have an incomplete CA cycle when growing anaerobically (Fig.2); the cycle serves as a biosynthetic pathway and the α-oxoglutarate effect may be considered an example of end-product inhibition.

(d) Other effectors

Inhibition of pig heart citrate synthase by palmitoyl-CoA has been reported by Wieland and Weiss (1963) and Srere (1965 b). Palmitoyl-CoA is a product of fatty acid synthesis and its effect was described by Srere (1968) as a non-specific detergent type of inhibition, probably of no regulatory importance. More recently Caggiano and Powell (1979) showed that a spin-labelled analogue of stearoyl-CoA inhibited citrate synthase at a specific allosteric fatty acyl binding site and concluded that there may be some physiological role in the inhibition, probably by regulating fatty acid biosynthesis through the levels of citrate.

Feedback inhibitions have also been observed by succinyl-CoA in rat heart mitochondria (Lanoue et al.,1972) and cyanobacteria (Lucas and Weitzman, 1977). In cyanobacteria an incomplete CA cycle is found due to the absence of α-oxoglutarate dehydrogenase. The incomplete CA cycle serves a dual biosynthetic role and the two end products α-oxoglutarate and succinyl-CoA of the branched pathway inhibit citrate synthase.

Two instances of AMP activation have been reported; these are for the enzymes from Azotobacter vinelandii

(Flechtner and Hanson, 1970) and a marine pseudomonad (Massarini and Cazzulo, 1974). Activation of the latter enzyme by inorganic phosphate has also been observed (Higa *et al.*, 1978). These activation effects are consistent with the control of citrate synthase by "energy charge".

Macnab *et al.* (1973) and Srere and Mosbach (1974) have shown that the microenvironment of an enzyme in a cell or organelle may differ markedly from the *in vitro* environment used for the study of the enzyme. All the studies of citrate synthase described above were carried out on cell-free extracts. In order to study the behaviour of citrate synthase *in situ* a number of investigations have been carried out using cells made permeable by treatment with toluene. The inhibitions of the bacterial enzymes by NADH and α-oxoglutarate were identical with those observed *in vitro* (Weitzman, 1973). However the inhibition by ATP *in situ* was absent in yeast (Weitzman and Hewson, 1973) and considerably reduced in rat liver (Matlib *et al.*, 1978). These results cast some doubt on the physiological significance of the *in vitro* effect.

Mutant Bacterial Citrate Synthases

One way to study the physiological significance of the various regulatory properties of citrate synthase is

to isolate mutant bacterial citrate synthases which have an altered response to effector metabolites. It may then be possible to examine any physiological dysfunction which has arisen and, at the same time, investigate the molecular properties of the modified enzyme.

Genetically altered enzymes are obtained by selecting mutant organisms which have an alteration in the DNA sequence (or gene) coding for a protein. Such mutants will produce a protein with a different primary structure. All the amino acids are susceptible to changes. The usefulness of this method is that only slight changes in structure, even the substitution of one amino acid, can result in a considerable change in the regulatory properties of the enzyme. Kantrowitz et al. (1980) isolated a series of single amino acid substitution mutants of aspartate carbamoyltransferase from E.coli and showed by kinetic studies that some of the mutants had altered regulatory properties.

Harford and Weitzman (1978) isolated mutants of E.coli citrate synthase. Citrate synthase-deficient strains were obtained by treatment with ethylmethanesulphonate and a number of spontaneous revertants, which had regained

citrate synthase activity, were isolated from these citrate synthase-deficient strains.

Three types of revertant citrate synthases were obtained (Table 1) and these resembled the diverse, naturally-occurring citrate synthases. Revertant type 1 citrate synthase was 'large' and resembled the wild-type enzyme, revertant type 2 citrate synthase was also 'large' but was insensitive to inhibition by NADH. This enzyme resembled the citrate synthase found in A.xylinum (Swissa and Benziman, 1976). Revertant type 3 citrate synthase was of the 'small' type and had kinetic and regulatory properties typical of a 'small' citrate synthase.

It appeared that the loss of subunit interactions in revertant enzyme types 2 and 3 accounted for the changes in response to substrates and inhibitors. The enzymes have been partially purified and some of their molecular and catalytic properties have been investigated (Harford, 1977). The immediate challenge is to devise a suitable means of purification so that further investigations of the molecular structures of these mutant enzymes can be undertaken.

Table 1

Comparison of some of the molecular, catalytic and regulatory properties of wild-type and revertant *E.coli* citrate synthases

Type of citrate synthase	Molecular weight	Substrate dependence	Km or $S_{0.5}$ for acetyl-CoA (μM)	Ki NADH (mM)
Wild-type	230,000	Sigmoidal	420	0.04
Revertant type 1	230,000	Sigmoidal	360	0.06
Revertant type 2	230,000	Hyperbolic	30.3	13.7
Revertant type 3	110,000	Hyperbolic	10.4	4.8

This thesis presents the results of some attempts to prepare an immobilized ligand for the affinity chromatography of citrate synthase. Particular attention has been paid to revertant type 2 citrate synthase which has been shown to be very unstable and present in low levels.

A further series of mutant citrate synthases has been isolated by Weitzman et al. (1978) from transformed Acinetobacter calcoaceticus. Citrate synthase-deficient strains requiring glutamate for growth were obtained by selection for resistance to fluoroacetate. Transforming DNA preparations from both the parent strain and Ps.aeruginosa were applied to the citrate synthase-deficient strains and cells transformed to prototrophy by regaining citrate synthase activity were selected. The transformant citrate synthases fell into two groups; in one group the enzymes were 'large', inhibited by NADH and deinhibited by AMP whereas the enzymes in the other group were 'small' and totally insensitive to NADH.

Thus by two separate methods of mutation on different organisms, families of 'large' and 'small' citrate synthases may be obtained and these should prove valuable in structure-function studies.

Professor P.D.J. Weitzman's research group is investigating the properties of a number of bacterial citrate synthases and it is the findings of an investigation into the properties of Ps.aeruginosa citrate synthase which are presented in this thesis.

Ps.aeruginosa is a strict aerobe and its citrate synthase is typical of the 'large' type of enzyme associated with this group of bacteria, i.e. it is inhibited by NADH and deinhibited by AMP. One interesting feature of the enzyme is that it is readily desensitized to NADH inhibition by DTNB without loss of enzyme activity (Weitzman and Danson, 1976). Another strict aerobe, A.calcoaceticus, has a citrate synthase which is insensitive to treatment with DTNB. This difference in behaviour of citrate synthases within a particular group of bacteria prompted a survey of the effect of a number of specific thiol-blocking reagents on the citrate synthases from different organisms.

A mutant Ps.aeruginosa was kindly provided by Professor Patricia H. Clarke. This mutant was reported as having low levels of citrate synthase (Skinner and Clarke, 1968) and it was thought that this citrate synthase-deficient strain could serve as a starter for the production of citrate synthase mutants in Ps.aeruginosa.

Such mutants would complement those of E.coli and Acinetobacter.

In the event, however, an examination of this mutant showed that two forms of citrate synthase were present in the same organism.

Characterization of the enzymes showed that one enzyme was of the 'large' type, NADH-sensitive and, rather unusually, sensitive to AMP activation. The other enzyme was 'small' and insensitive to both NADH and AMP. Furthermore, the ratio of the two enzyme forms was found to be dependent on the growth state of the batch culture. When the organism was grown on nutrient broth the 'large' citrate synthase was found to predominate in a log-phase culture whereas the 'small' citrate synthase was found to predominate in a stationary-phase culture with no apparent loss of activity of the 'large' enzyme.

The unusual features of the mutant provide an opportunity to compare 'large' and 'small' citrate synthases and complement the investigations of other citrate synthases (wild-type and mutant) being carried out in this laboratory.

MATERIALS AND METHODS

MATERIALS

Chemicals

Citrate synthase (pig heart), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart), NADP-linked isocitrate dehydrogenase (pig heart), oxaloacetate and CoASH were from Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.; catalase (bovine liver), β-galactosidase (Escherichia coli), Sepharose 4B, chloramphenicol, dithiothreitol, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2,2'-dithiobis-(5-nitropyridine) (DTNP), 2,2'-dipyridyl disulphide (2-PDS), 4,4'-dipyridyl disulphide (4-PDS), ATP, ADP, AMP, GDP, NADPH, NADH, $NADP^+$, NAD^+, adipic acid dihydrazide, cyanogen bromide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), α-oxoglutarate and DL-isocitrate were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; Sephadex G-200 and G-25, AH-Sepharose 4B, CH-Sepharose 4B, Activated CH-Sepharose 4B, Activated Thiol-Sepharose 4B, Sepharose 4B CNBr-activated and 5'-AMP-Sepharose 4B were obtained from Pharmacia (Great Britain) Ltd., London, U.K.; Methylene Blue, Rose Bengal and sodium periodate were from BDH Chemicals Ltd., Poole, U.K.; Matrex Gel Red A was obtained from Amicon Ltd., High Wycombe, Buckinghamshire, U.K.; dephospho-CoASH was

obtained from P-L Biochemicals Inc., Milwaukee, U.S.A.; DEAE-cellulose (Whatman grade DE-11) was from H.Reeve Angel and Co., London, U.K. and protamine sulphate was from Koch-Light Laboratories Ltd., Colnbrook, U.K. All other chemicals used were analytical grade or the finest grade commercially available.

Preparation of Acetyl-CoA and Succinyl-CoA

Acetyl-CoA was prepared by the acetylation of CoASH with acetic anhydride (Stadtman, 1957). Succinyl-CoA was similarly prepared using succinic anhydride.

Organisms

The pseudomonad organisms used in this work were Pseudomonas aeruginosa (NCIB 8295) and a mutant, Pseudomonas aeruginosa 8602 (At 14), isolated by Skinner and Clarke (1968).

The strains of Escherichia coli used in this work are listed in Table 2 which also shows the auxotrophic markers they carry. The abbreviations used for genetic markers are those of Taylor and Trotter (1972).

Other organisms used were Acinetobacter calcoaceticus, strain 4B (isolated by P.D.J. Weitzman) and Bacillus megaterium (laboratory strain).

Table 2

Strains of *E.coli* used in this work

Strain	Markers	References
AB1621	F^- *ara*, *lac*, *gal*, *thi*, str^r	Ashworth *et al.*(1965)
AB1623.R6	F^- *ara*, *lac*, *gal*, *thi*, str^r, $gltA^{rev}$	Harford (1977)
AB1623.R9	F^- *ara*, *lac*, *gal*, *thi*, str^r, $gltA^{rev}$	Harford (1977)

r = resistant

rev = revertant

METHODS

Maintenance of Organisms

Organisms were stored at 4°C on nutrient agar slopes supplemented with 10 mM-glutamate. All strains were maintained by periodic subculture. Organisms in frequent use were also stored as single colony isolates on nutrient agar plates supplemented with 10 mM-glutamate.

Media

Organisms were grown on either nutrient broth supplemented with 10 mM-glutamate (hereafter referred to as nutrient-glutamate) or the basal salts medium of Ashworth and Kornberg (1966) with a carbon source at a concentration of 10 mM unless otherwise stated. Nitrogen-free media were obtained by replacing the 0.5 M-NH_4Cl with double distilled water.

Sterilization was carried out in an autoclave at 15 lb/in^2 (103.5 kPa) for 20 min.

Growth of Organisms

Liquid cultures were obtained by inoculating 200 ml of media with a pure culture of the organism from a

nutrient agar plate. The cultures were grown in Erlenmeyer flasks on a rotary shaker at 37°C. To ensure aeration the volume of liquid never exceeded 40% of the volume of the containing vessel.

Growth was monitored by measuring the increase in turbidity at 680 nm. Samples were added to a 3 ml cuvette of 1 cm lightpath and the absorbance was measured with a Unicam SP6 spectrophotometer. The samples were diluted with fresh medium to obtain an absorbance reading of less than 0.25. At readings above this value there was a non-linear relationship between the number of cells and absorbance.

Samples (5 ml) of overnight (16 h) cultures were transferred to 2.5 l flasks containing 1 litre of fresh medium and the cultures were allowed to grow throughout the day. Exponentially-growing cultures were harvested at an absorbance of 0.1 and were called log-phase cultures (see Fig.3). Stationary-phase cultures were harvested when there was no further increase in absorbance which was usually at an absorbance of about 1.7.

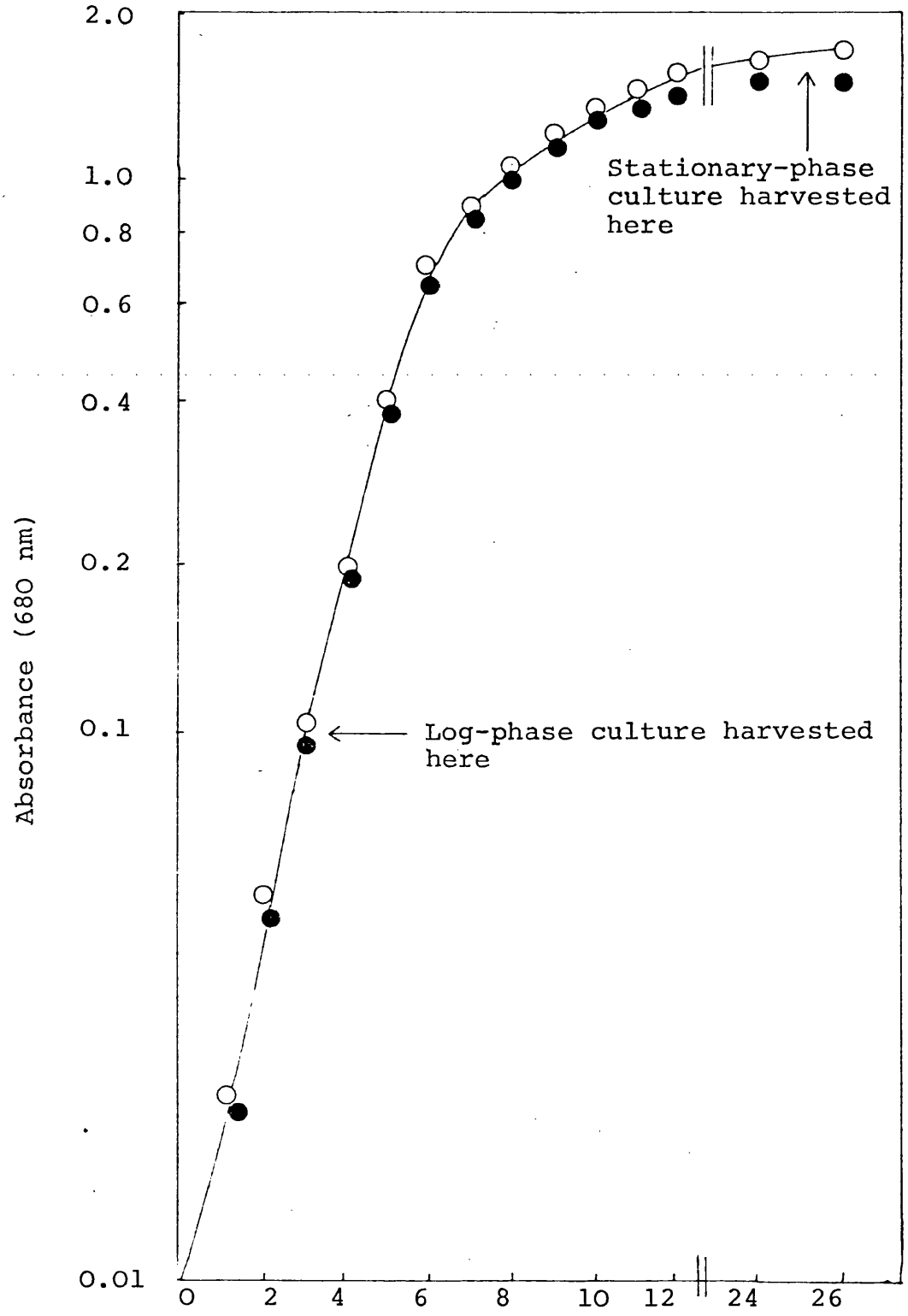


Fig.3. Growth curve of Ps.aeruginosa on nutrient-glutamate at 37°C

5 ml of an overnight (16 h) culture were transferred to 1 litre of fresh medium and growth monitored at 680 nm.

(○) wild-type Ps.aeruginosa;
(●) mutant Ps.aeruginosa.

Enzyme Assays

1. Citrate Synthase

Two methods were used to assay this enzyme.

Method 1

Citrate synthase was assayed spectrophotometrically at 412 nm and 25°C by the method of Srere et al. (1963) with a Unicam SP1800 spectrophotometer coupled to a Unicam AR 25 recorder. Unless otherwise stated, assay mixtures contained 20 mM-Tris-HCl (pH 8.0), 1 mM-EDTA (hereafter called 'Tris buffer (pH 8.0)'), 0.2 mM-oxaloacetate, 0.15 mM-acetyl-CoA and 0.1 mM-DTNB.

The reaction was started by the addition of enzyme to bring the total volume to 1.0 ml and was followed by monitoring the appearance of the thionitrobenzoate anion (TNB^-) which has a molar absorbance of 1.36×10^4 at pH 8.0 and 412 nm (Ellman, 1959).

Method 2

Citrate synthase was also measured by the continuous polarographic method of Weitzman (1969 b). The reaction vessel contained 0.2 mM-oxaloacetate and

0.15 mM-acetyl-CoA in 'Tris buffer (pH 8.0)'. Nitrogen was bubbled through for a few minutes to expel all oxygen. The reaction was started by the addition of enzyme to bring the total volume to 1.0 ml. The production of CoASH in the reaction vessel resulted in the appearance of an anodic current which was monitored at -0.3 volt versus a saturated calomel electrode. Calibration of the mercury electrode showed that 1.0 mM-CoASH produced a wave of 1.7 μA at this potential.

The advantage of the polarographic method over the spectrophotometric method is that it does not require DTNB, which inactivates certain citrate synthases, and it enables assays to be conducted over a wider range of pH.

Enzyme Units

One unit of enzyme is defined as that quantity producing 1 μmole of CoASH/min under standard assay conditions and specific activity is defined as enzyme units/mg of protein.

Estimation of Substrate Concentrations

The concentration of acetyl-CoA was determined by assay Method 1. The oxaloacetate and DTNB concentrations

were in approximately three-fold excess over the acetyl-CoA. Pig heart citrate synthase (10 μg) was added and the reaction allowed to proceed to completion. The concentration of acetyl-CoA was calculated from the increase in absorbance at 412 nm. The concentration of oxaloacetate was similarly determined with excess amounts of acetyl-CoA and DTNB.

2. Lactate Dehydrogenase

This enzyme was measured by monitoring the oxidation of NADH at 340 nm and 25°C (Kornberg, 1955 a). The reaction mixture contained 0.2 mM-NADH and 0.2 mM-pyruvate. The reaction was started by addition of enzyme to give a total volume of 1.0 ml. The molar absorbance of NADH was taken to be 6.2×10^3.

3. Malate Dehydrogenase

The oxidation of NADH was monitored at 340 nm and 25°C in the presence of 0.2 mM-NADH and 0.2 mM-oxaloacetate. The reaction was started by the addition of enzyme to give a final volume of 1.0 ml (Ochoa, 1955). The molar absorbance of NADH was taken to be 6.2×10^3.

4. β-Galactosidase

The hydrolysis of 0.5 mM-o-nitrophenyl β-D galactoside was monitored at 405 nm and 25°C in a total volume of 1.0 ml (Wallenfels, 1962). The molar absorbance of o-nitrophenol was taken to be 3.1×10^3.

5. Catalase

This enzyme was measured by its absorbance at 400 nm (Weitzman and Dunmore, 1969 a).

6. Isocitrate Dehydrogenase

This enzyme was assayed by the determination of $NADP^+$ reduction at 340 nm (Kornberg, 1955 b). Reaction mixture contained 40 mM-phosphate buffer (pH 7.0), 3 mM-$MgCl_2$, 1.5 mM-$NADP^+$ and 0.2 mM-DL-isocitrate. The molar absorbance of NADPH was taken to be 6.2×10^3.

7. Succinyl-CoA Synthetase

This enzyme was assayed by the continuous polarographic method of Weitzman (1969 b). The reaction mixture contained 0.1 M-phosphate (pH 8.0), 10 mM-$MgCl_2$, 0.15 mM-succinyl-CoA and 0.5 mM-ADP. In the case of the enzyme from A.calcoaceticus, GDP was substituted

for ADP. Calibration of the mercury electrode showed that 1 mM-CoASH produced a wave of 1.7 μA at a potential of -0.3 volt.

Rapid Determination of Molecular Size of Different Forms of Citrate Synthase

1. Preparation of cell-free extracts

Cells were grown as previously described. Two litres of a log-phase culture of Ps.aeruginosa were harvested at 23,000 g for 10 min. The pellet was then washed with 20 mM-Tris-HCl (pH 7.0), 1 mM-EDTA (hereafter referred to as 'Tris buffer (pH 7.0)') containing 0.1 M-KCl. The cells were resuspended in as small a volume of buffer as possible and sonicated at full power (180 watts) using a Rapidis Ultrasonic Oscillator for a total of 2 min. The sonication was conducted in intermittent bursts with cooling in ice-water to prevent overheating of the extract. Cell debris was removed by centrifugation at 38,000 g for 15 min. This and all subsequent steps were carried out at $4^{o}C$.

The same procedure was applied to a stationary-phase culture; however, only 1 litre of cells was harvested. Total citrate synthase activity was measured using assay Method 1 and the protein estimated by the

method of Lowry *et al.* (1951) with bovine serum albumin as standard.

2. Gel filtration on Sephadex G-200

A volume of cell-free extract not greater than 1 ml containing 0.01 ml (10 µg) of the marker protein rabbit muscle lactate dehydrogenase was made 10% (w/v) in sucrose and applied to a column (25.0 x 1.5 cm) of Sephadex G-200 equilibrated in 'Tris buffer (pH 7.0)' containing 0.1 M-KCl. Elution was performed with the same buffer at a flow rate of 10-15 ml/h, 1-ml fractions being collected.

Weitzman and Dunmore (1969 *a*) showed that 'large' citrate synthases (mol.wt.≃250,000) have a molecular weight greater than lactate dehydrogenases (mol.wt. 140,000), and should therefore be eluted from the column before this marker protein. Conversely 'small' citrate synthases (mol.wt.≃100,000) should be eluted from the column after lactate dehydrogenase.

Purification of Wild-Type *Ps.aeruginosa* Citrate Synthase

The purification was based on the method described by Weitzman (1969 *a*).

Step 1. Preparation of cell-free extracts

A 30 g (10 l) batch of frozen cells obtained from a stationary-phase culture grown on nutrient broth was thawed, washed and suspended to a total volume of 50 ml in 'Tris buffer (pH 7.0)' containing 0.1 M-KCl and 0.1 mM-dithiothreitol (DTT). This suspension was passed through a French press at 12,000 lb/in^2 (8.3 x 10^4 kPa) and cell debris was removed by centrifugation at 38,000 g for 30 min. This and all subsequent steps were carried out at 4^{o}C.

Step 2. Treatment with protamine sulphate

An aqueous 2% (w/v) solution of protamine sulphate was added slowly to the supernatant (1 ml of protamine sulphate per 10 mg of protein) and the mixture stirred for 15 min. The precipitate was removed by centrifugation at 38,000 g for 20 min.

Step 3. Heat treatment at 60^{o}C

The supernatant was heated to 60^{o}C for 5 min in a waterbath in the presence of 0.2 mM-NADH. The flask was immediately cooled to 4^{o}C. Cell debris was removed by centrifugation at 38,000 g for 20 min.

Step 4. Treatment with ammonium sulphate

Finely-ground ammonium sulphate was added to the supernatant until the solution was 50% saturated. The mixture was stirred for 15 min and the precipitate removed by centrifugation. More ammonium sulphate was added to the supernatant until the solution was 70% saturated and the mixture again stirred for 15 min. The precipitate was collected by centrifugation and dissolved in 250 ml of 'Tris buffer (pH 7.0)'.

Step 5. DEAE - cellulose chromatography

The diluted enzyme was applied to a column (35.0 x 2.5 cm) of DEAE-cellulose previously equilibrated with 'Tris buffer (pH 7.0)'. The column was washed with 'Tris buffer (pH 7.0)' containing 50 mM-KCl until no more protein was eluted. A linear gradient of KCl (500 ml) from 0.05 to 0.5 M in 'Tris buffer (pH 7.0)' was then applied and the effluent collected in 5-ml fractions. Citrate synthase was eluted at about 0.18 M-KCl. The most active fractions were pooled and finely-ground ammonium sulphate was added until the solution was 70% saturated. The precipitate was collected and dissolved in a small volume of 'Tris buffer (pH 7.0)' containing 0.1 M-KCl.

Step 6. Gel filtration

The solution was made 10% (w/v) in sucrose, applied to a column (35.0 x 2.5 cm) of Sephadex G-200 previously equilibrated with 'Tris buffer (pH 7.0)' containing 0.1 M-KCl, and the enzyme eluted with the same buffer; 2-ml fractions were collected. Protein estimations of purified preparations were derived from the values of absorbance at 260 and 280 nm (Layne, 1957).

Overall, a 500-fold purification was obtained (Table 3).

Purification of the Mutant Ps.aeruginosa Citrate Synthase

The procedure was similar to the one described above, with the omission of the heat step. Two peaks of enzyme activity were obtained from the Sephadex G-200 column. One peak corresponded to a 'large' enzyme (hereafter referred to as CS I) and the other peak corresponded to a 'small' enzyme (hereafter referred to as CS II). The most active fractions of the two enzymes were separately pooled. The following additional step was then inserted.

Step 6. Matrex Gel Red A

CS II was diluted to 50 mM-KCl and applied to a small column of Matrex Gel Red A (volume 5 ml) previously

Table 3

Purification of wild-type Ps.aeruginosa citrate synthase

Step	Volume (ml)	Total Enzyme Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	Recovery (%)
1. French-press extract	50	294	2125	0.14	-
2. Protamine sulphate supernatant	58	298	1856	0.16	101
3. Thermal precipitation	52	191	624	0.31	65
4. 50-70% satd. (NH_4) SO_4 precipitate	250	165	200	0.83	56
5. Pooled selected fractions from DEAE-cellulose column	10	130	5.7	22.8	44
6. Pooled selected fractions from Sephadex G-200 column	12	61	0.9	68	20

equilibrated with 'Tris buffer (pH 7.0)'. The column was washed with buffer until no more protein was eluted. The enzyme was then eluted with a combination of 0.1 mM-CoASH and 0.1 mM-oxaloacetate in 'Tris buffer (pH 7.0)'; 0.5-ml fractions were collected. Protein estimations at 260 and 280 nm were impossible due to the high absorbance of the CoASH at these wavelengths. The best fractions were pooled and dialysed overnight against 'Tris buffer (pH 7.0)' containing 0.1 M-KCl to enable protein estimations to be made by the method of Layne (1957).

CS I was applied to another Matrex Gel Red A column. No elution was possible with various combinations of CoASH and oxaloacetate. Elution was achieved with 'Tris buffer (pH 7.0)' containing 0.2 M-KCl.

Overall, a 100-fold purification of CS II and a 50-fold purification of CS I were obtained (Table 4).

Polyacrylamide-Gel Electrophoresis

Disc gel electrophoresis at pH 8.9 was performed according to the method of Davis (1964) with 7% polyacrylamide gels. Fifty µg of purified protein was

Table 4

Purification of mutant Ps.aeruginosa citrate synthase

Step		Volume (ml)	Total Enzyme Activity (units)	Total Protein (mg)	Specific Activity (units/mg protein)	Recovery (%)
1. French-press extract		150	363	1500	0.24	-
2. Protamine sulphate supernatant		152	386	1175	0.33	106
3. 40-70% satd. $(NH_4)_2SO_4$ precipitate		200	294	610	0.48	80
4. Pooled selected fractions from DEAE-cellulose column		50	187	70	2.67	51
5. Pooled selected fractions from Sephadex G-200 column	CS I	18	4.2	7.3	0.55	1.2
	CS II	34	84	41	2.05	23
6. Pooled selected fractions from Matrex Gel Red A	CS I	3	3.6	1.1	3.17	0.9
	CS II	3	54	3.1	17.42	15

applied to each gel and, after electrophoresis, protein was detected by staining with 1% Coomassie Brilliant Blue in 12.5% trichloracetic acid by the method of Chrambach et al. (1967).

The enzyme was not purified to homogeneity; another step was therefore needed to determine which of the protein bands corresponded to enzyme activity. The citrate synthase bands were located by cutting the gel into 1.5 mm slices; each slice was placed on a white spotting tile and broken up in 0.2 ml 'Tris buffer (pH 8.0)', with a glass rod; a visual estimation of enzyme activity was then made using the principle of assay Method 1. In this way Rf values for the enzymes were calculated.

Preparation of Immobilized Ligands for Affinity Chromatography

Procedure 1. CNBr-activated Sepharose

CNBr-activated Sepharose was prepared from Sepharose 4B according to the method of Axen et al. (1967). Fifteen grams packed weight of Sepharose were washed with 500 ml of distilled water and then resuspended in 150 ml of distilled water. Cyanogen bromide solution (15 g/300 ml H_2O) was added to the stirred suspension in a fume cupboard and the reaction mixture maintained at 10^{o}-20^{o}C and pH 11-12 by adding 2 M-NaOH. The suspension was

filtered on a sintered glass filter and then washed with 500 ml of cold 0.1 M-$NaHCO_3$, 500 ml of cold distilled water and, finally, 250 ml of 0.1 M-sodium acetate (pH 6.0). The resultant CNBr-activated Sepharose 4B was used immediately.

CoASH was attached to the matrix according to the method of Chibata et al. (1974) (see Fig.4). CNBr-activated Sepharose was added to a solution of CoASH (25 mg) in 0.1 M-sodium acetate (pH 6.0). The mixture was gently stirred for 5 h at 25°C, filtered and washed with 50 ml of 1 M-NaCl. The resultant immobilized CoASH derivative was washed with 50 ml of aqueous mercaptoethanol (0.5 ml/50 ml H_2O) to provide the reduced form and then washed with 0.1 M-sodium acetate (pH 6.0) until the washings no longer produced an absorbance at 412 nm with DTNB. The gel was then washed with 0.1 M-Tris buffer (pH 7.5) and a check made that the mixture gave a yellow colour with DTNB. Concentrated acetic anhydride (0.03 ml) was then added and left stirring for 20 min until the coenzyme was acetylated and no yellow colour was produced with DTNB.

The amount of acetyl-CoA immobilized was determined by the method of Canovas and Kornberg (1966). A portion

(0.02 ml) of suspended gel was added to 0.3 ml of 1 mM-DTNB and this was made up to a final volume of 2.85 ml with distilled water. After standing at room temperature for 5 min the absorbance was read at 412 nm. Freshly prepared 4M-hydroxylamine hydrochloride was then mixed with 4 M-KOH and 0.15 ml of the resultant solution was added to the above gel mixture and the absorbance again read at 412 nm after 15 min at room temperature. The difference between the readings corresponded to the CoASH formed from the immobilized acetyl-CoA.

Procedure 2. CH-Sepharose and carbodiimide (Mosbach et al., 1972)

CH-Sepharose is a matrix containing a 6-carbon spacer with a free carboxyl group. The spacer is believed to relieve steric hindrance. CH-Sepharose (500 mg) was washed with 200 ml of 0.5 M-NaCl on a glass sinter for 15 min and then suspended in 2 ml of distilled water (pH 4.5).

CoASH (4 mg) was dissolved in 1 ml of distilled water (pH 4.5) and, together with 20 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), also in 1 ml of distilled water (pH 4.5),was added to the

gel and stirred overnight at room temperature (Fig.5). The gel was then washed with 1 M-NaCl and the CoASH acetylated as described in procedure 1.

Procedure 3. Activated CH-Sepharose (according to "Affinity Chromatography, Principles and Methods")

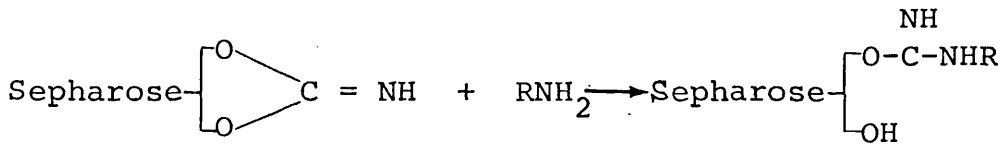
Acetyl-CoA was attached directly to Activated CH-Sepharose through the 6-amino group of the adenine residue (Fig.6).

Activated CH-Sepharose (0.5 g) was washed and reswelled on a glass sinter with 100 ml of 1 mM-HCl. CoASH (10 mg) was dissolved in 1 ml of 0.1 M-$NaHCO_3$ (pH 8.0) and acetylated with acetic anhydride. Acetyl-CoA was then mixed with the gel and stirred for 1 h at room temperature. Excess ligand was washed away with coupling buffer and excess active groups blocked by adding 0.1 M-Tris (pH 8.0) for 1 h. The gel was then washed with 0.05 M-Tris (pH 8.0), 0.5 M-NaCl and finally with 0.05 M-formate (pH 4.0), 0.5 M-NaCl.

Procedure 4. Activated Thiol-Sepharose

CoASH was immobilized through its thiol group to Activated Thiol-Sepharose (Fig.7). Activated Thiol-Sepharose (0.5 g) was washed in 100 ml of 20 mM-Tris

Fig.4. Attachment of CoASH through the 6-amino group to CNBr-activated Sepharose



CNBr-activated Sepharose isourea derivative

Fig.5. Attachment of CoASH to CH-Sepharose through the 6-amino group

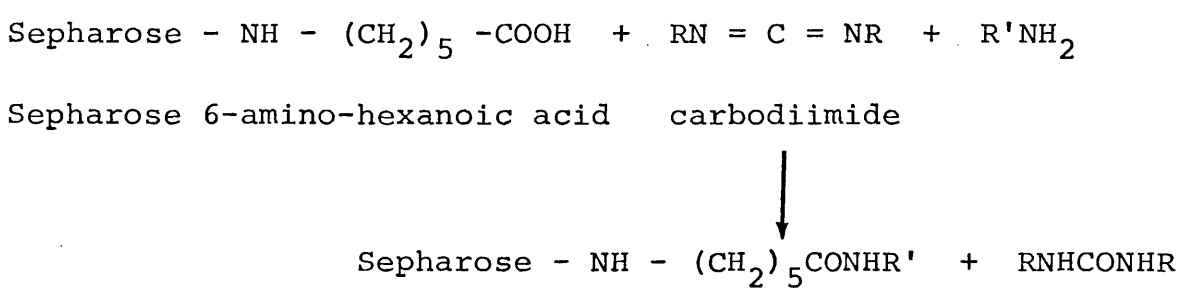


Fig.6. Attachment of Acetyl-CoA to Activated CH-Sepharose through the 6-amino group

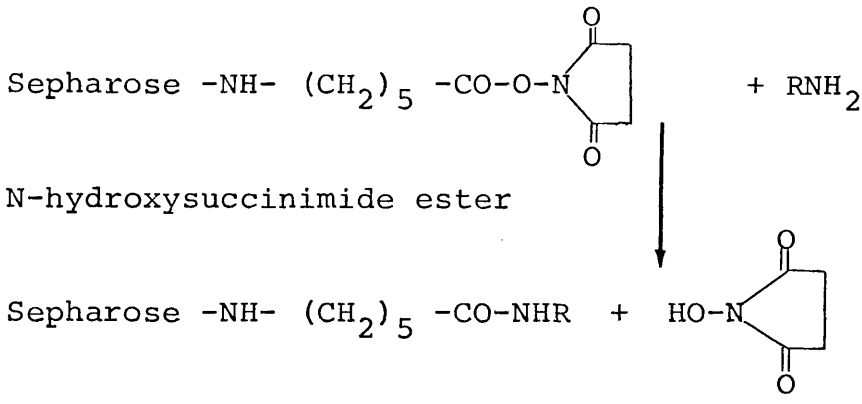
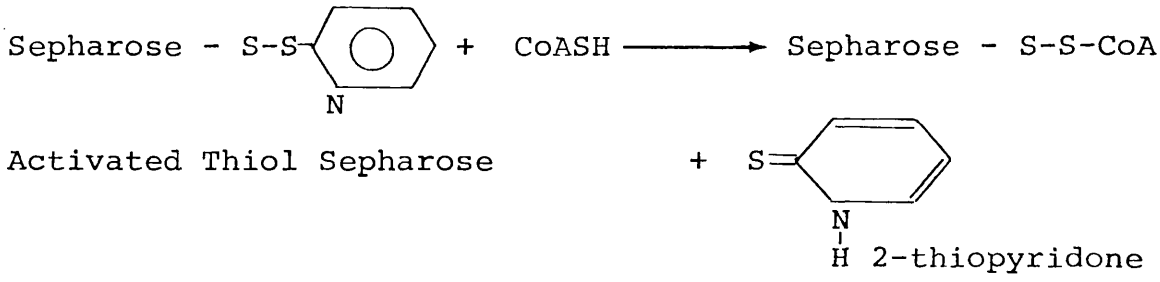


Fig.7. Attachment of CoASH through the thiol group to Activated Thiol-Sepharose



(pH 7.0), 1 mM-EDTA for 15 min and then collected on a sinter and washed with the same buffer. CoASH (4 mg) was dissolved in 5 ml of 0.1 M-Tris (pH 8.0) and added to the swollen gel. This was then stirred overnight with a magnetic stirrer at room temperature. The amount of CoASH coupled was determined by measuring the amount of 2-thiopyridone released in the effluent at 343 nm (molar absorbance = 8.08×10^3).

Procedure 5. AH-Sepharose and carbodiimide (Cuatrecasas, 1970)

Oxaloacetate, α-oxoglutarate, citrate and isocitrate were immobilized on AH-Sepharose through their carboxyl groups (Fig.8). According to the manufacturer, AH-Sepharose contains 6-10 μmoles of spacer group/ml of swollen gel.

AH-Sepharose (0.5 g) was reswelled on a glass sinter with 100 ml of 0.5 M-NaCl and added to 2 ml (40 μmoles) of ligand (pH 4.5) and 2 ml (84 mg) of EDC (pH 4.5). This was stirred overnight at room temperature. Acid-base titration curves were used to show whether the ligand was bound to the matrix. One ml of thoroughly washed gel was mixed with 1 ml of distilled water. Titration curves from pH 2-10 were plotted using 0.01 ml samples of 0.1 M-HCl and 0.1 M-NaOH.

Procedure 6. Various nucleotides were attached to agarose hydrazide through the OH groups in the 2'-3' position of the ribose moiety according to the method of Lamed et al. (1973).

(a) Preparation of Sepharose 4B-adipic acid dihydrazide conjugate (Fig.9)

One g of CNBr-activated Sepharose 4B was reswelled on a glass sinter with 200 ml of 1 mM-HCl and then added to 3.5 ml of cold saturated adipic acid dihydrazide (90 gl^{-1}) in 0.1 M-sodium carbonate buffer (pH 9.5). This was left stirring overnight at 4°C and then washed thoroughly with distilled water and 0.2 M-NaCl.

(b) Periodate-oxidation of nucleotides

One ml of 0.02 M-cold neutral nucleotide was mixed with 1 ml of 0.02 M-sodium metaperiodate (Gilham, 1971) and oxidation allowed to proceed for 1 h in the dark at 4°C.

(c) Binding of periodate-oxidised nucleotides to Sepharose-adipic acid hydrazide (Fig.10)

To 2 ml of Sepharose hydrazide in 0.1 M-sodium acetate (pH 5.0) was added 10 µmoles of periodate-oxidised nucleotide in 5 ml of the same buffer. This was stirred

Fig.8. Attachment of ligands through their carboxyl groups to AH-Sepharose

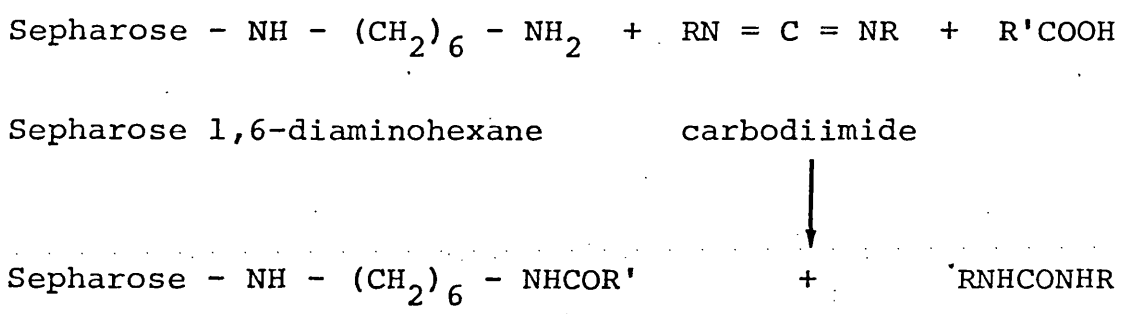


Fig.9. Attachment of adipic acid dihydrazide to CNBr-activated Sepharose

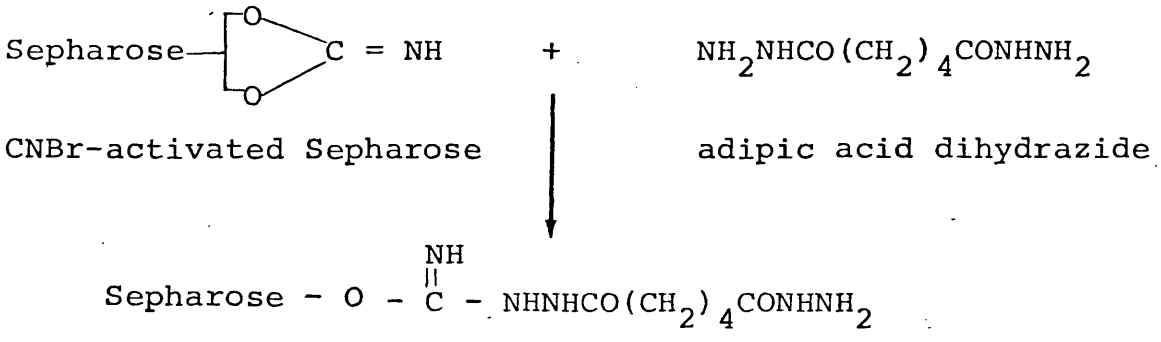
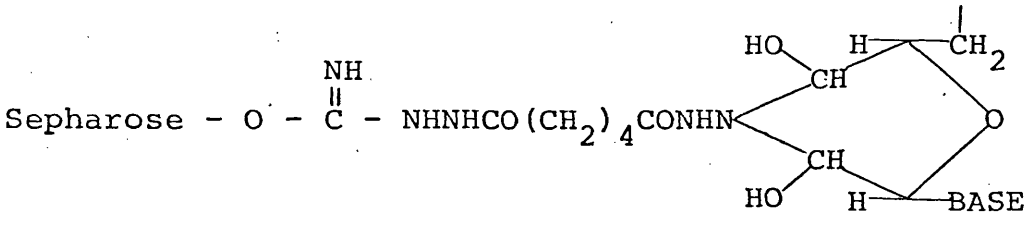


Fig.10. Periodate-oxidised nucleotide coupled to Sepharose-adipic acid dihydrazide



for 3 h at 4°C. At the end of this period 15 ml of 2 M-NaCl were added and the mixture stirred for a further 30 min. The column was finally washed with excess distilled water.

(d) Reduction of NAD^+-Sepharose (Barry and O'Carra, 1973)

Sodium dithionite (250 mg) was added to 1 ml of nucleotide gel and suspended in 20 ml of 1.3% $NaHCO_3$. Nitrogen (99.9% oxygen-free) was bubbled through the suspension which was then sealed and incubated at 15°C for 2 h with continuous stirring. The gel was then washed on a sintered glass funnel with 100 volumes of 0.2 M-NaCl and 10 volumes of distilled water.

Adsorption and elution of citrate synthase

Small columns (2-3 ml) were poured at 4°C and first equilibrated with 'Tris buffer (pH 8.0)'. Ten-fold diluted pure pig heart citrate synthase was applied to a Sephadex G-25 column to remove ammonium sulphate and collected in 0.5-ml fractions. The most active fractions were pooled. A sample of enzyme (0.2 ml containing 40 µg of protein) was applied to the affinity column as a quick test and 1-ml fractions were collected. If the enzyme bound, elution was attempted and, if successful, the procedure

was repeated with a protamine sulphate treated extract of a bacterial citrate synthase. A variety of substances was used to elute the enzyme including 0.1 M-KCl, 1 mM-oxaloacetate, 1 mM-acetyl-CoA, 1 mM-ATP and the mixture 0.1 mM-CoASH plus 0.1 mM-oxaloacetate used by Mukherjee and Srere (1976) to form dead-end complexes with the enzyme.

Thermal Inactivation Studies of Citrate Synthase

Citrate synthase (0.2 ml) was incubated in 'Tris buffer (pH 8.0)' at various temperatures in a waterbath for 5 min. Aliquots (0.05 ml) were then withdrawn and assayed for activity.

In addition, the enzyme was incubated at a fixed temperature in the presence of various effectors to see if they affected thermal stability.

Dye-Sensitized Photo-Oxidation of Citrate Synthase

Photo-oxidation was carried out with a 150 watt spot-light positioned 20 cm from the reaction mixture contained in a glass conical centrifuge tube immersed in water maintained at 20^{o}C. Into the tube were placed 0.4 ml of 'Tris buffer (pH 8.0)', 0.05 ml of 30 µM-photo-sensitive dye and 0.05 ml of enzyme solution (10 µg protein).

At intervals, samples were withdrawn and immediately diluted five-fold with 0.1 M-Tris-HCl (pH 8.0) in small glass tubes covered with aluminium foil, to prevent further reaction. Suitable control experiments were carried out in the absence of dye or light.

Treatment of Citrate Synthase with Specific Thiol-Blocking Reagents

Citrate synthase was incubated in 1.0 ml of 'Tris buffer (pH 8.0)' with a number of specific thiol-blocking reagents at 20°C. At intervals samples were removed and measurements were made of the activity and, where appropriate, the inhibition by NADH. The following reagents were used:

(1) 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)
(2) 2,2'-dithiobis-(5-nitropyridine) (DTNP)
(3) 2,2'-dipyridyl disulphide (2-PDS)
(4) 4,4'-dipyridyl disulphide (4-PDS)

Analysis of Data

Km values were determined by entering enzyme velocity measurements and various substrate concentrations into program I (Appendix).

Values of $S_{0.5}$ were obtained using computer program II (Appendix).

RESULTS

Section A. Growth-Phase Dependent Citrate Synthases

Growth of Wild-Type Ps.aeruginosa on Different Media

Ps.aeruginosa is a very versatile organism and is able to grow on a large number of different carbon sources (Stanier et al., 1966). Often a lag period was observed when a culture from one medium was transferred to a different carbon source. This was presumably due to the induction of specific permeases necessary for the movement of the substrate across the cell wall (Kogut and Podoski, 1953; Clarke and Meadow, 1959). A variation of growth rate was observed on different batches of nutrient broth. This was probably due to a slight change in the constituents of the nutrient and by supplementing the medium with 10 mM-glutamate a reproducible growth curve was obtained.

There was very little variation in the level of citrate synthase in Ps.aeruginosa when grown on different carbon sources (Table 5). However, in all cases there was an increase in specific activity from a log-phase culture to a stationary-phase culture. Only one form of citrate synthase was obtained by elution from a Sephadex G-200 column (Fig.11). The enzyme was eluted before the marker lactate dehydrogenase which corresponded

Table 5

Levels of citrate synthase on different carbon sources

Organism	Carbon source 10 mM		Specific Activity of citrate synthase (units/mg protein) Log-phase culture	Stationary-phase culture
Wild-type *Ps.aeruginosa*	Nutrient*-glutamate		0.112	0.324
	Acetate		0.141	0.337
	Glucose		0.172	0.421
Mutant *Ps.aeruginosa*	Nutrient*-glutamate	CS I	0.006	0.025
		CS II	0.001	0.375
	Acetate	CS I	0.032	0.063
		CS II	0.174	0.348
	Glucose	CS I	0.063	0.222
		CS II	0.081	0.511

*Nutrient broth 13 gl^{-1}

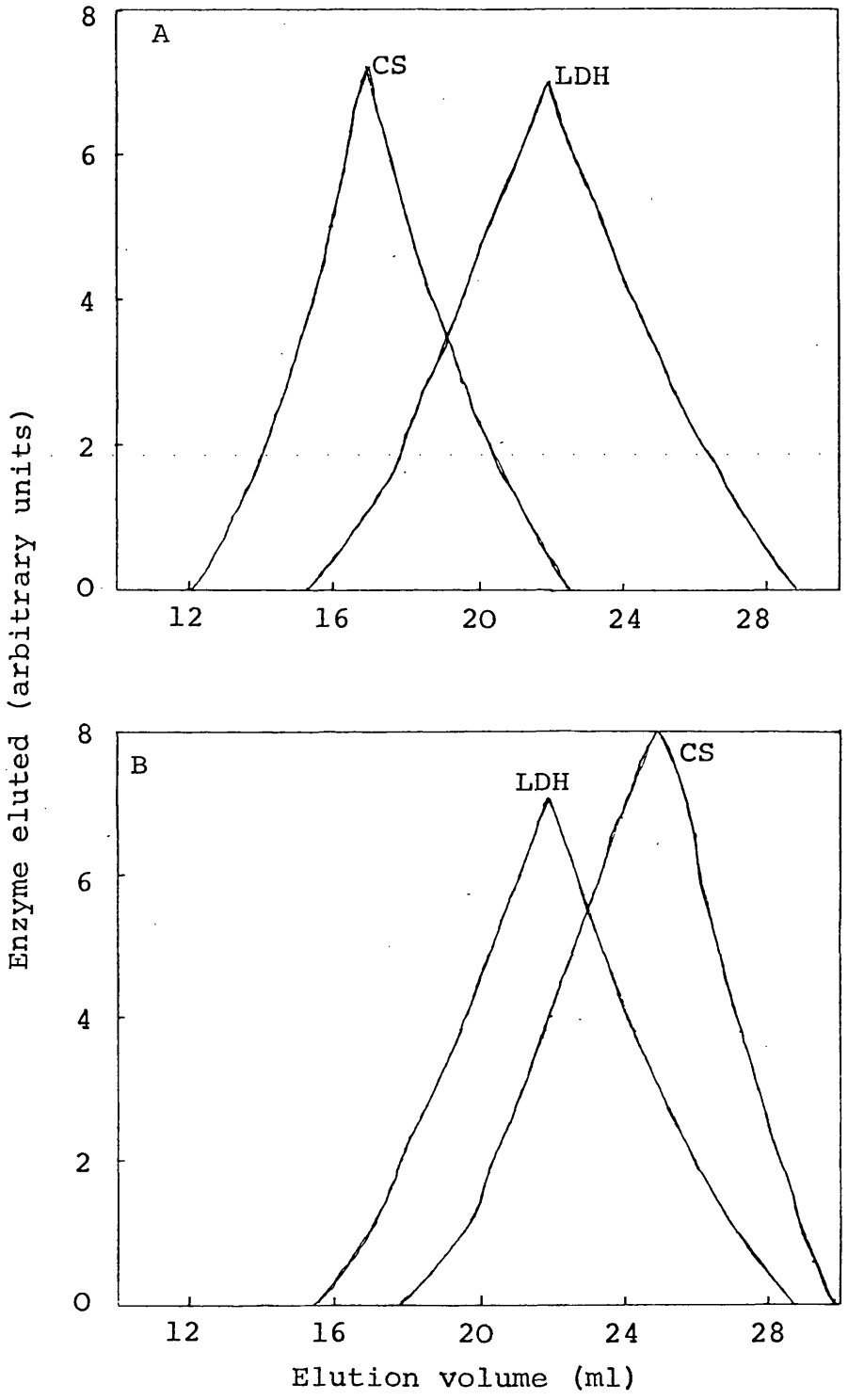


Fig.11. Elution profile of citrate synthase and lactate dehydrogenase on a Sephadex G-200 column
Buffer: 'Tris buffer (pH 7.0)', 0.1 M-KCl.
A 'large' wild-type Ps.aeruginosa citrate synthase.
B 'small' pig heart citrate synthase.
CS, citrate synthase; LDH, lactate dehydrogenase.

to the 'large' form of citrate synthase (Weitzman and Dunmore, 1969 a). Pig heart citrate synthase, a 'small' enzyme, was eluted after lactate dehydrogenase.

Growth of Mutant Ps.aeruginosa on Nutrient-Glutamate

Two forms of citrate synthase were found in the mutant Ps.aeruginosa when grown on nutrient-glutamate. In the log phase of growth a 'large' form of citrate synthase predominated (CS I) whereas, in stationary phase, a 'small' form of citrate synthase predominated (CS II) (Fig.12).

When both forms of the enzyme were present in similar amounts (late log phase) it was difficult to separate them completely on a Sephadex G-200 column. In order to differentiate the two forms of the enzyme a method based on the AMP activation ratio was devised. CS I was activated by 0.5 mM-AMP, maximal activation being observed when assayed in 'Tris buffer (pH 7.0)'; CS II was not activated by AMP. Thus by assaying all column fractions in the presence and absence of AMP it was possible to determine the amount of the two enzymes by a simple computation of the AMP activation ratio (Fig.13).

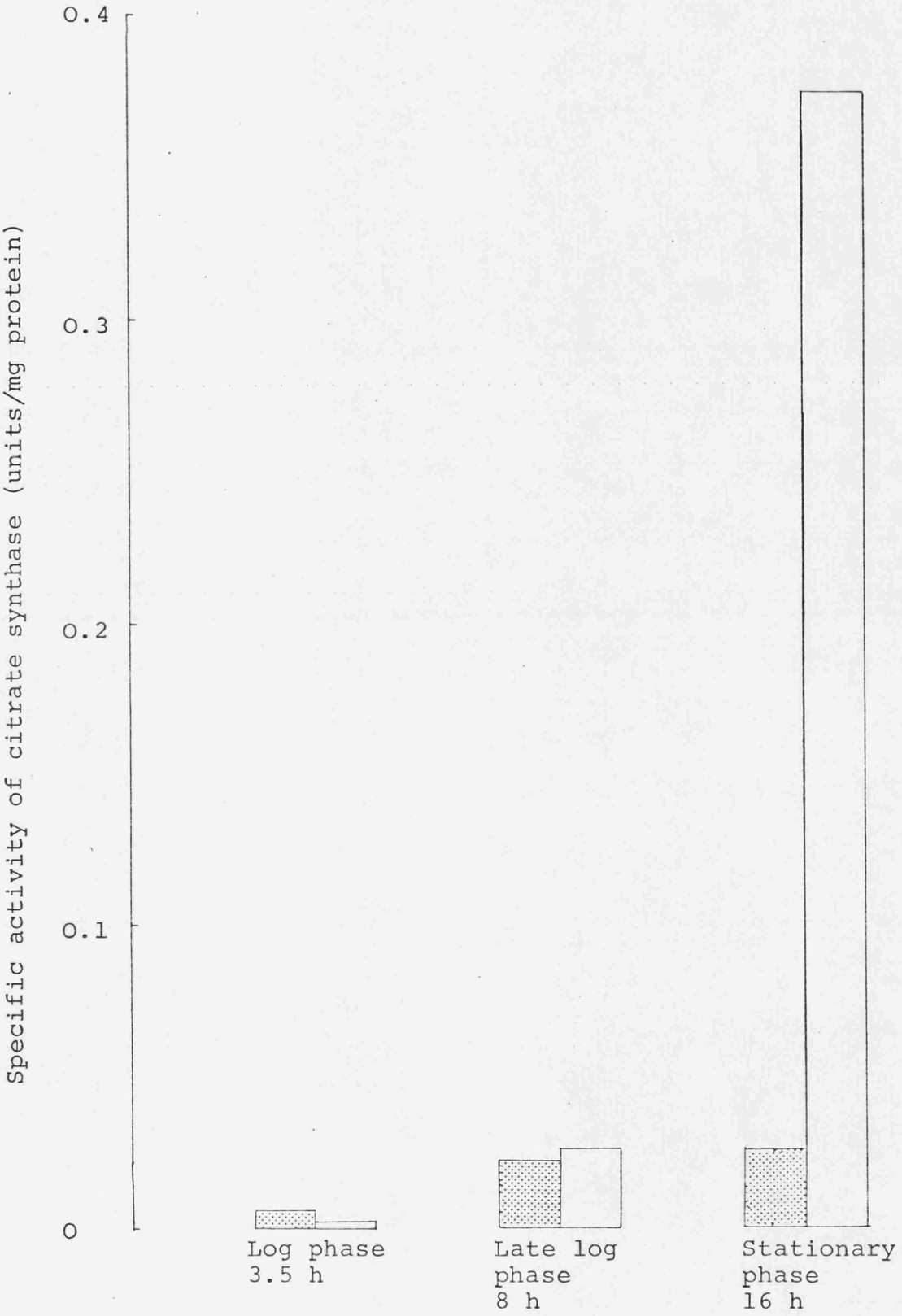


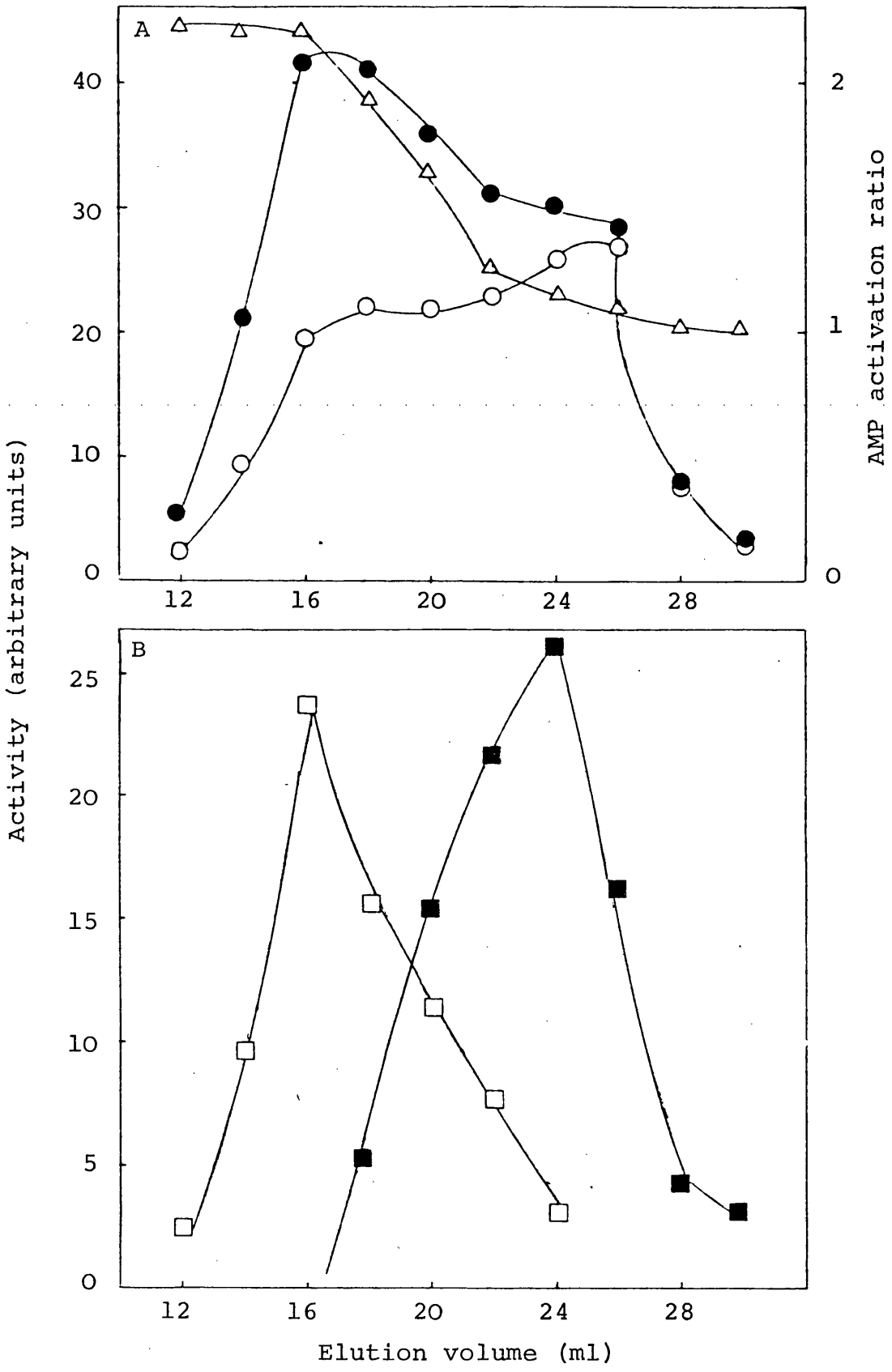
Fig.12. Histogram showing changes in levels of citrate synthase in mutant Ps.aeruginosa on nutrient-glutamate at 37°C

▨ CS I, □ CS II.

Fig.13. Separation of CS I and CS II of mutant Ps.aeruginosa by gel filtration on Sephadex G-200
Crude extract applied to column in 'Tris buffer (pH 7.0)', 0.1 M-KCl.

A (○) Enzyme activity in the absence of AMP;
(●) Enzyme activity in the presence of 0.5 mM-AMP;
(△) AMP activation ratio.

B (□) Enzyme activity of CS I obtained by a computation of the AMP activation ratio;(■)CS II.


A
B
Activity (arbitrary units)
AMP activation ratio
Elution volume (ml)
0
10
20
30
40
5
15
25
1
2
12
16
20
24
28

A larger Sephadex G-200 column could not be used to obtain better resolution of the two enzyme forms because very low levels of enzyme were found in a log-phase culture and it would have been difficult to detect enzyme activity in the fractions obtained from a larger column due to the dilution of the enzyme during passage through the column.

A change in pH of the medium was observed during growth on nutrient-glutamate. In log phase the pH was 7.0 and in late log phase the pH had increased to 7.4. The pH had risen to 8.0 in an overnight stationary-phase culture. In order to check that the change in pH was not responsible for the change in the ratio of CS I to CS II during growth, the organism was grown in a chemostat growth vessel with the pH maintained at 7.0. The changes in enzyme levels were identical to those previously described. Thus the pH of the medium was not directly responsible for the alteration of the ratio of CS I to CS II.

Both enzyme forms were present in cultures obtained from different single colonies, containing exclusively Gram-negative bacteria. This eliminated the possibility that CS II might be derived from a contaminant. In

addition, the growth curves of both wild-type and mutant Ps.aeruginosa were identical (Fig.3). One might expect an altered growth curve if a contaminant were present.

When separated forms of CS I and CS II were re-run on different Sephadex G-200 columns no association or dissociation of the enzymes was apparent, i.e. the separated enzymes re-ran as single peaks. The time period of sonication of the cells was also varied from 20 sec to 3 min. This had no effect on the ratio of CS I to CS II. Disruption of cells with the French press also failed to alter the enzyme levels. This suggested that the two forms of the enzyme were not an artifact of the breakage procedure.

Massarini and Cazzulo (1975) showed that dialysis of the larger form of citrate synthase from a marine pseudomonad against 20 mM-potassium phosphate buffer (pH 7.0) containing 1 mM-EDTA resulted in loss of AMP activation and almost total dissociation to the smaller form of citrate synthase. Partial reassociation was obtained by dialysis against 50 mM-Tris-HCl buffer (pH 7.0) (Higa et al., 1978). When these experiments were repeated with CS I and CS II from the mutant

Ps.aeruginosa no dissociation or reassociation was observed.

Treatment with a Protein Synthesis Inhibitor

Treatment of a growing culture with the antibiotic chloramphenicol (200 µg/ml) resulted in a rapid arrest of growth (Fig.14). Chloramphenicol binds reversibly to the 50S subunit of the bacterial ribosome and prevents protein synthesis (Nierhaus and Nierhaus, 1973). Transfer of a chloramphenicol-treated culture to fresh medium should result in normal growth. Fig.15 shows the effect of chloramphenicol on the levels of CS I and CS II after 6 h. There was no increase in CS II suggesting that CS II was not a breakdown product of CS I and that it was formed by de novo protein synthesis, unless chloramphenicol prevented synthesis of a proteolytic enzyme which reacted with CS I. Cultures treated with chloramphenicol overnight (16 h) were found to have started growing again. Prolonged treatment may have led to selection of a chloramphenicol-resistant form of mutant Ps.aeruginosa.

Effect of Age of Colonies from Nutrient Agar Plates on Levels of CS I and CS II

The age of a colony on a nutrient-glutamate agar plate also affected the ratio of CS I to CS II in a

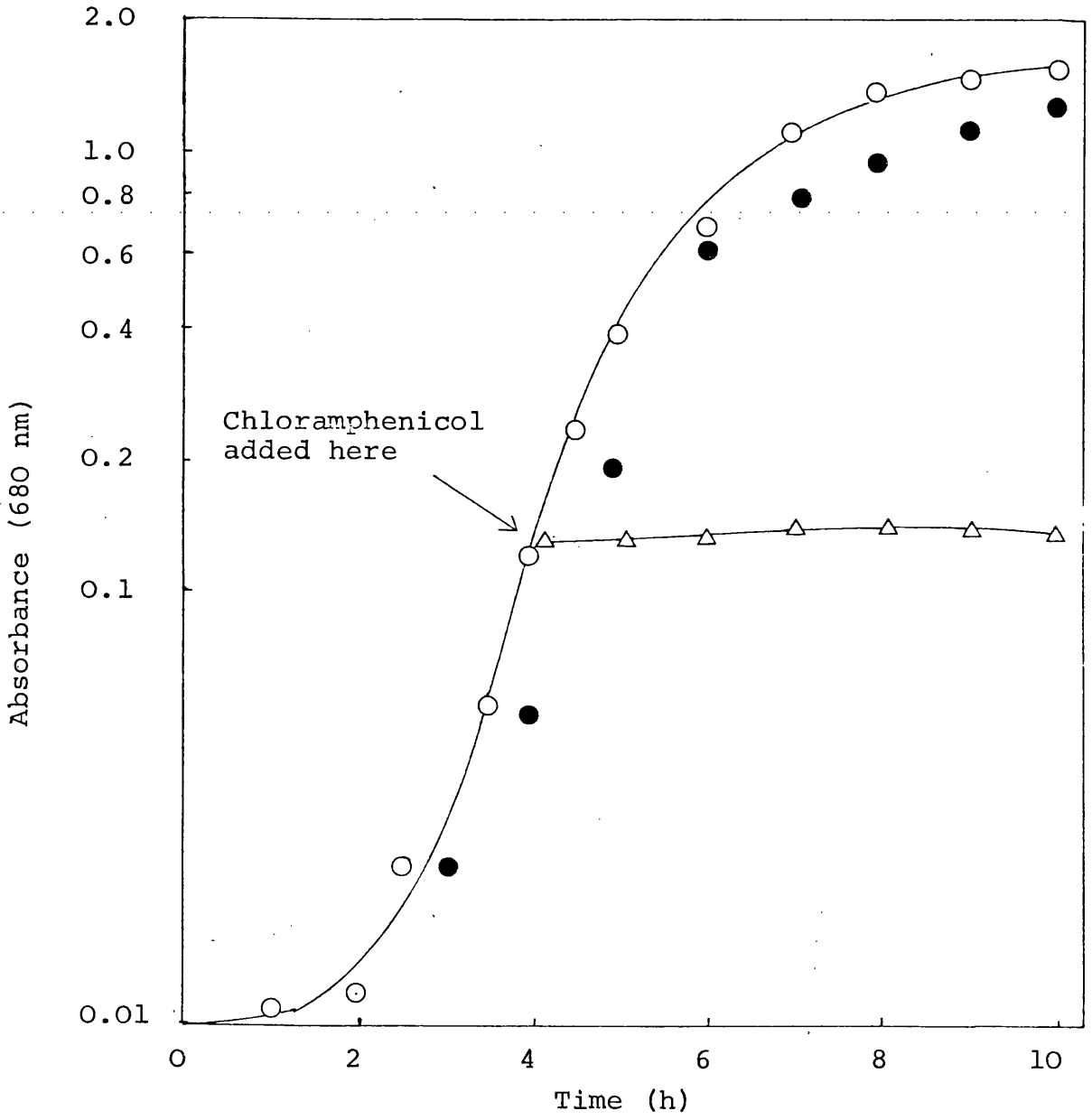


Fig.14. Effect of chloramphenicol on the growth rate of mutant Ps.aeruginosa on nutrient-glutamate at 37°C

(○) mutant Ps.aeruginosa,
(△) mutant Ps.aeruginosa + chloramphenicol (200µg/ml)
(●) mutant Ps.aeruginosa + chloramphenicol transferred to fresh medium.

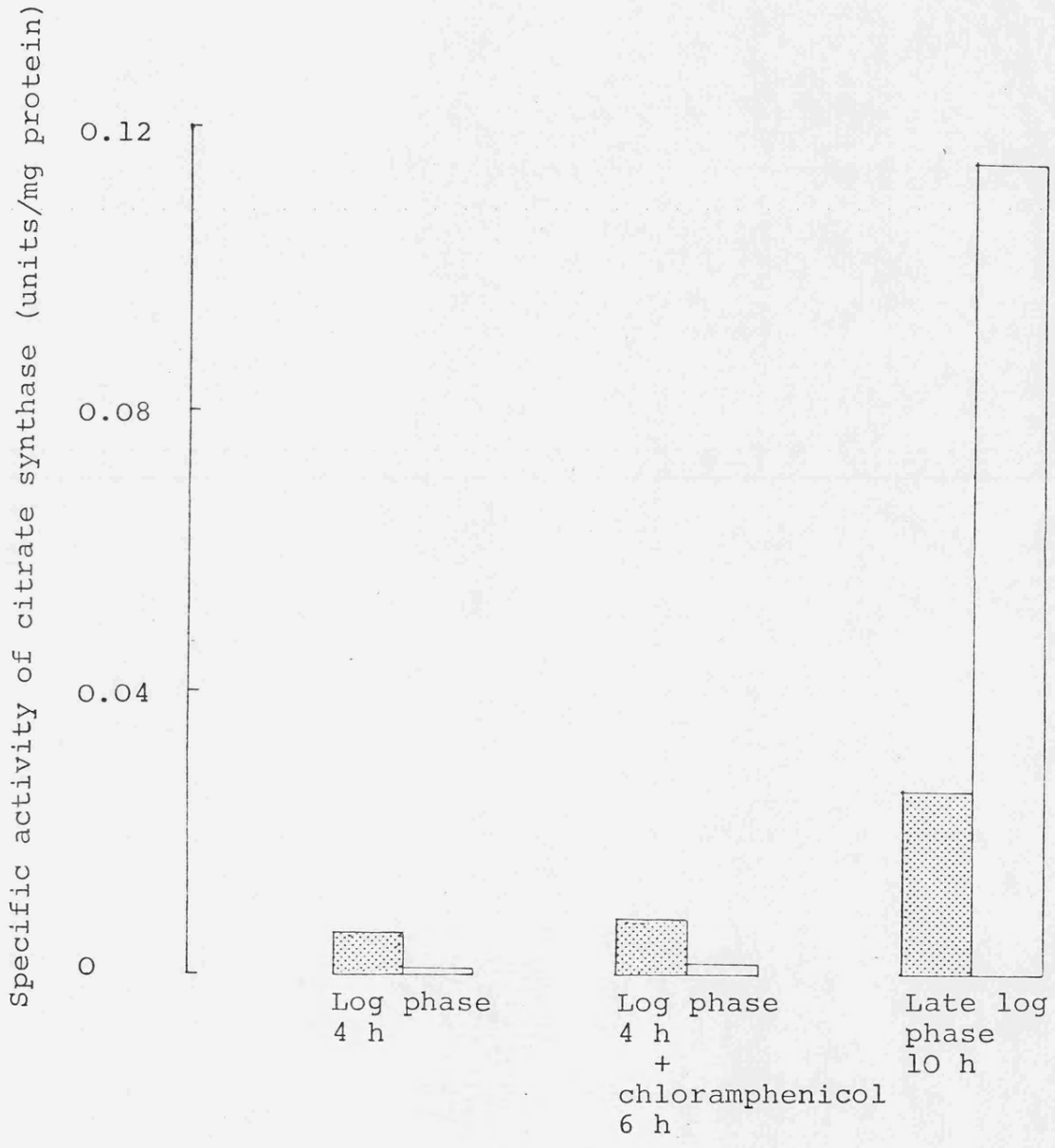


Fig.15. Histogram showing effect of chloramphenicol (200 µg/ml) on levels of citrate synthase in mutant Ps.aeruginosa grown on nutrient-glutamate at 37°C

▨ CS I, □ CS II.

log-phase liquid culture. The older the colony, the more CS II was obtained in log phase (Fig.16). The predominance of CS I in a log-phase culture could be 'restored' by repeated subculturing of an aged colony. The original level of CS I could be maintained by weekly subculturing the organism. The need for frequent subculturing was not fully appreciated in the earlier stages of this work and consequently a number of experiments were carried out with cultures obtained from plates which were over a week old. This did not in any way diminish the value of the experiments because changes in ratio of CS I to CS II were always observed when going from a log-phase culture to a stationary-phase culture.

Growth of Mutant Ps.aeruginosa on Spent Medium

The mutant Ps.aeruginosa was grown on nutrient-glutamate for 9 h until it was approaching stationary phase. The cells were harvested when the absorbance was 1.05 at 680 nm and the supernatant spent medium was resterilized. Five ml of fresh culture were added to the spent medium, growth was continued and the cells were harvested in log phase. There was very little change in the levels of CS I and CS II in log phase between fresh medium and spent medium (Fig.17).

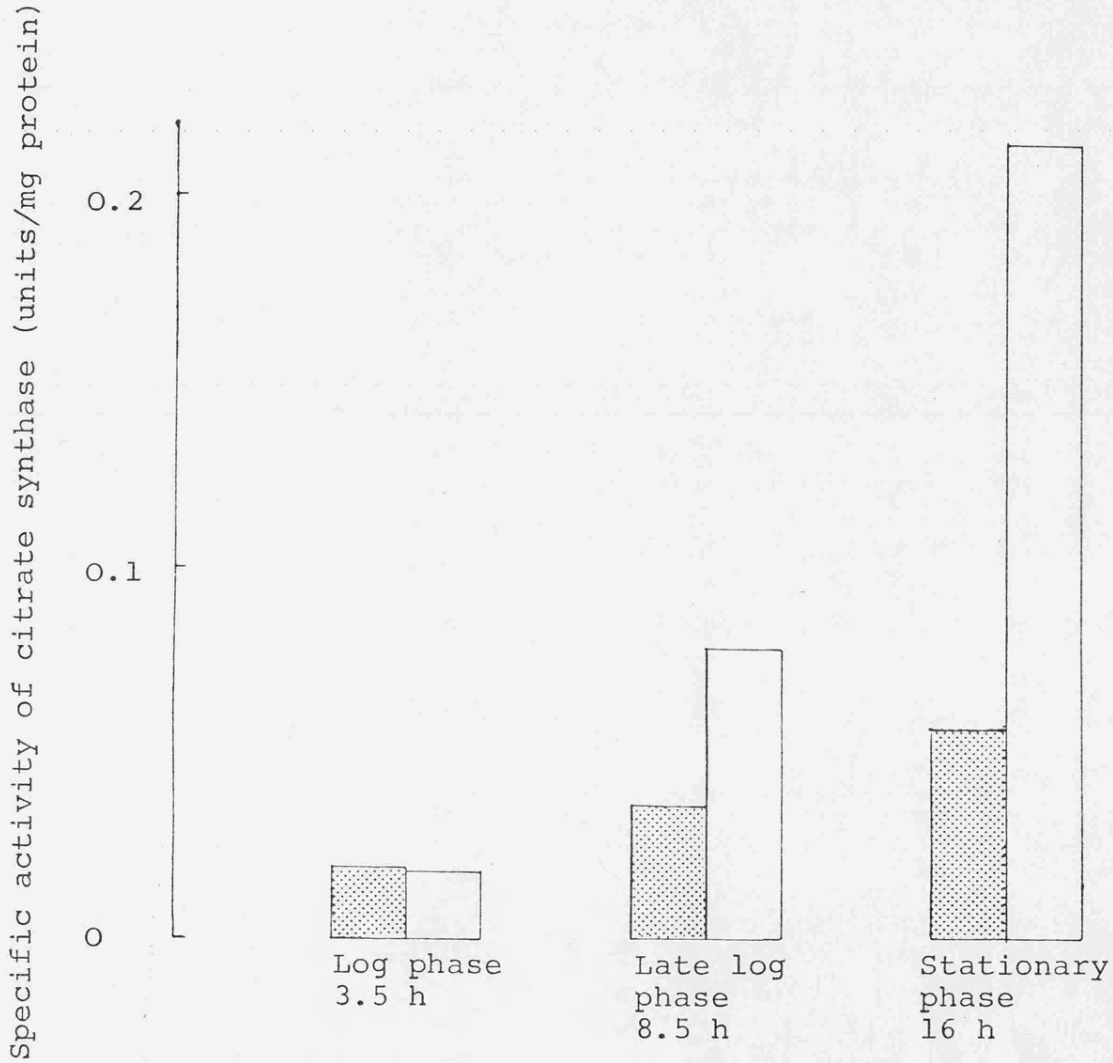


Fig.16. Histogram showing changes in levels of citrate synthase when a single colony of mutant Ps.aeruginosa from a 14 day old nutrient agar plate was grown on nutrient-glutamate liquid medium at 37°C

▒ CS I, □ CS II.

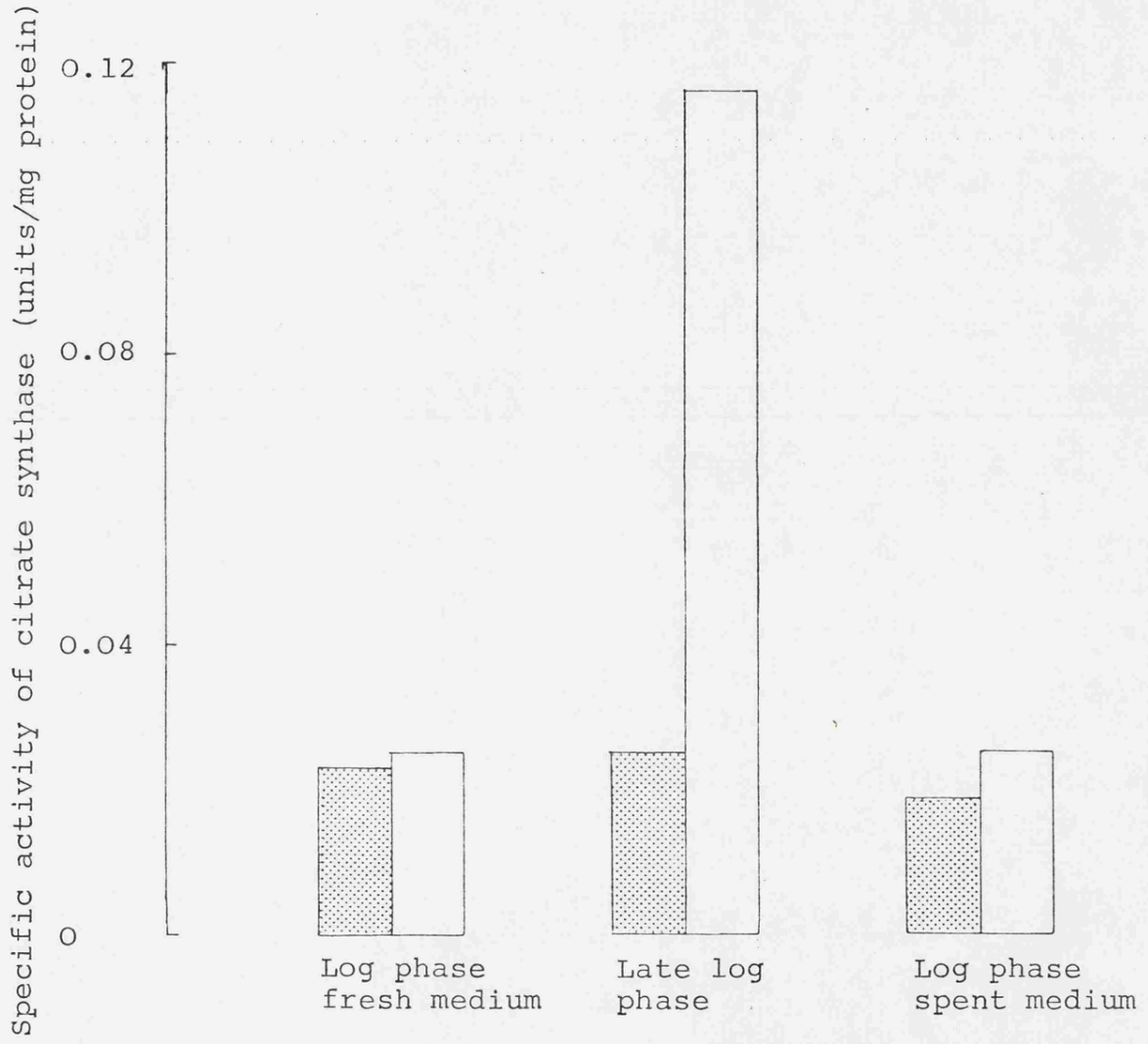


Fig.17. Histogram showing changes in levels of citrate synthase in mutant Ps.aeruginosa when grown on nutrient-glutamate and the medium then reused to support growth of a fresh culture

▨ CS I, □ CS II.

It was therefore unlikely that there was an uptake of a compound by the mutant from the medium when approaching stationary phase which resulted in increased synthesis of CS II.

Growth of Mutant Ps.aeruginosa on Different Carbon Sources

There was considerable variation in the levels of CS I and CS II when grown on different carbon sources (Table 5). This prompted a more detailed study and the results of growth on a number of different carbon sources are presented in Figs.18 and 19. The length of time a liquid culture was left in stationary phase did not alter the levels of CS I and CS II. For instance, the ratio of CS I to CS II when grown on 10 mM-glucose was the same if the cells were harvested after 1 day or 1 week.

Differences in growth rate were also observed on different carbon sources (Table 6). There was no correlation between doubling time and levels of CS I and CS II in a log-phase culture. The amount of carbon available may have affected the levels of citrate synthase in a culture. There was three times as much carbon available with 10 mM-glucose compared with 10 mM-acetate. The organism was also grown on 30 mM-acetate. There was no change in doubling time and the levels of citrate

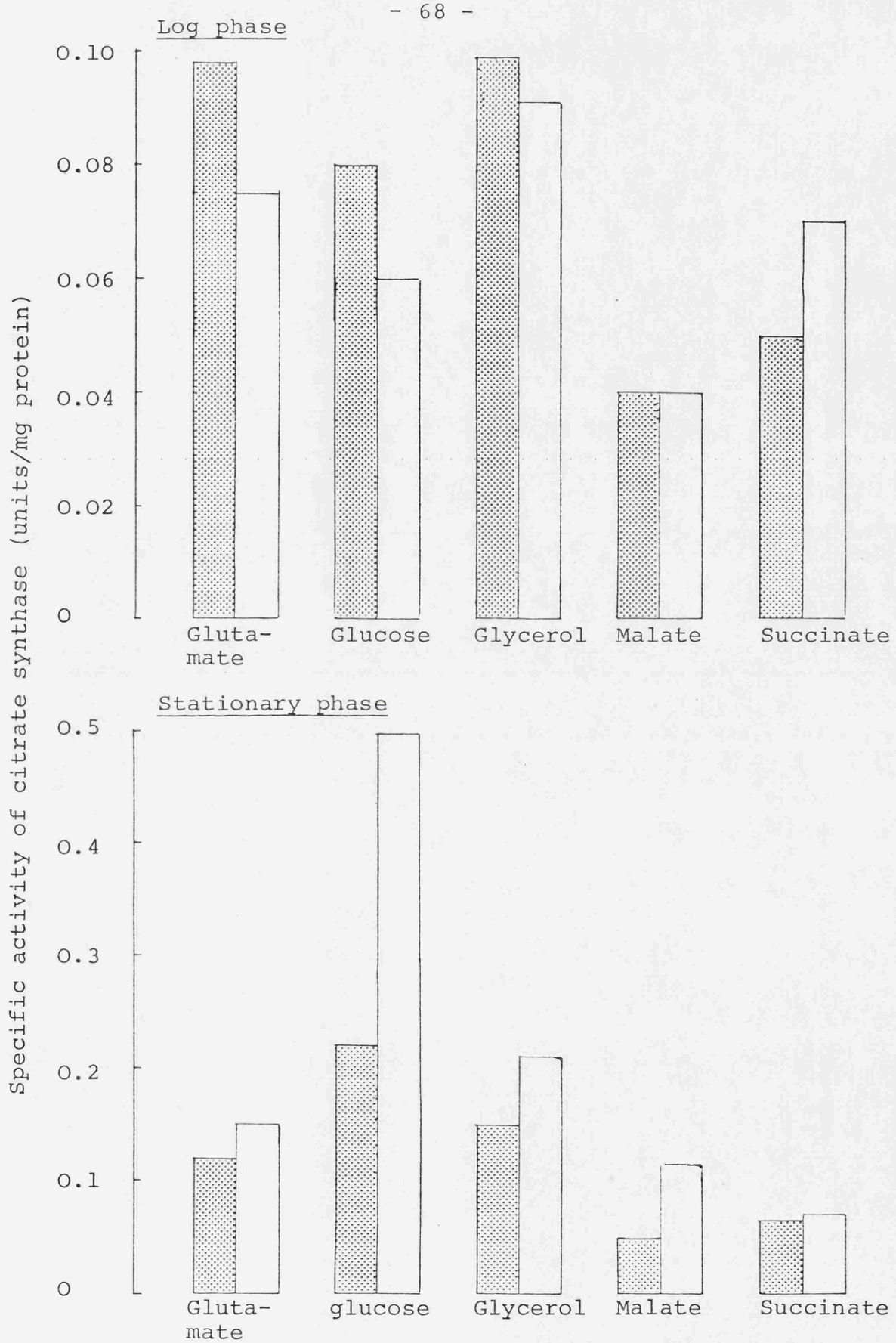


Fig.18. Variation of levels of citrate synthase in mutant Ps.aeruginosa grown on different carbon sources (10 mM)

▨ CS I, □ CS II.

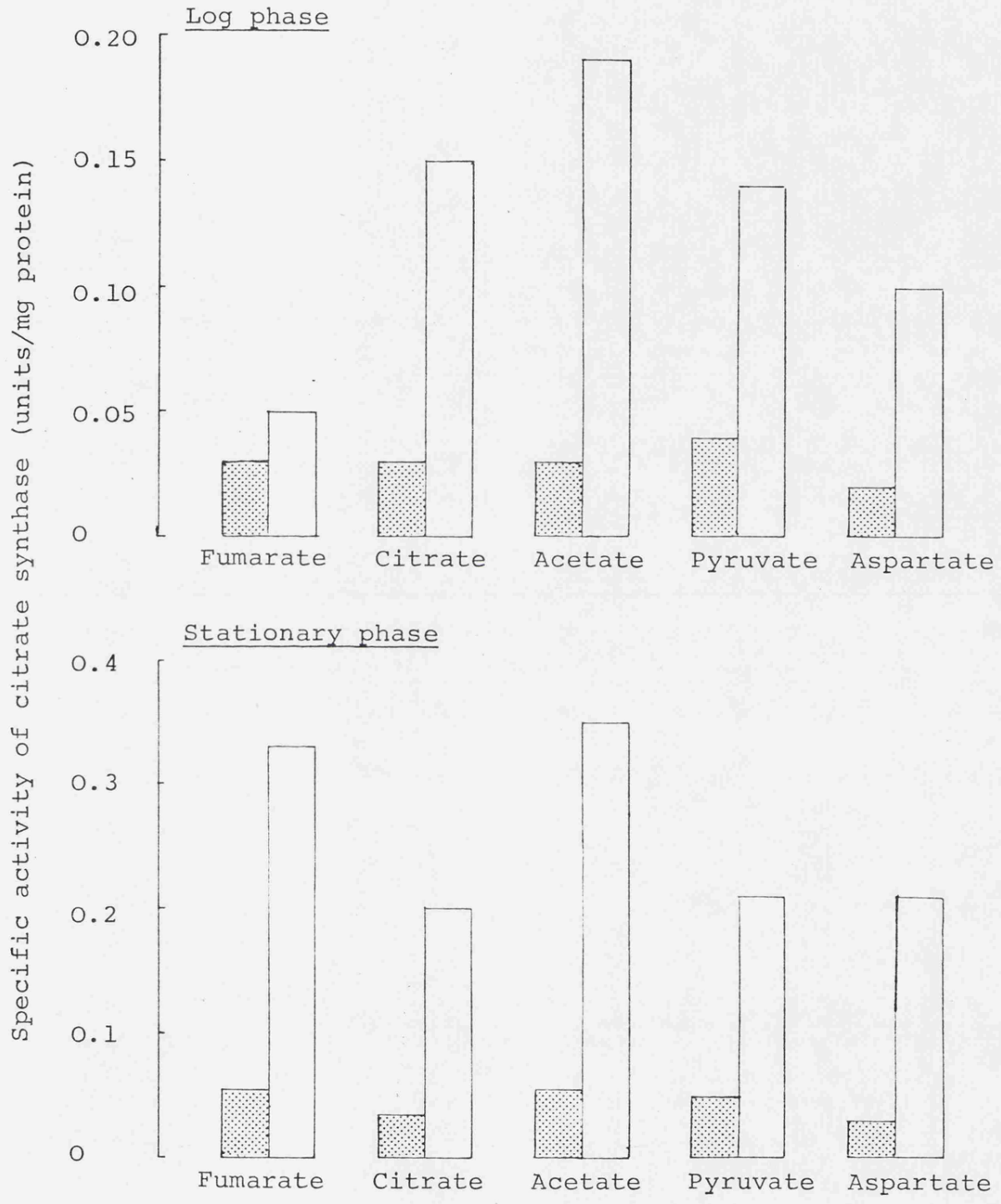


Fig.19. Variation of levels of citrate synthase in mutant Ps.aeruginosa grown on different carbon sources (10 mM)

▨ CS I, □ CS II.

Table 6

Variation of the doubling times of mutant Ps.aeruginosa on different carbon sources in liquid medium

Carbon source	Doubling time (min)
Acetate	45
Acetate*	45
Aspartate	65
Citrate	55
Fumarate	55
Glutamate	70
Glucose	55
Glycerol	175
Malate	40
Pyruvate	65
Succinate	60

The concentration of the carbon source was 10 mM except in the case of acetate*, when it was 30 mM.

synthase were identical at both concentrations.

Transfer of 10 ml of a stationary-phase culture grown on 30 mM-acetate to 1 litre of nutrient-glutamate resulted in the production of the levels of CS I and CS II one would expect when a log-phase culture on nutrient-glutamate was examined; there was a predominance of CS I. This precluded the possibility that growth on different carbon sources resulted in selection of different mutants.

Effect of Nitrogen-Free Medium on Levels of CS I and CS II

Four litres of mutant Ps.aeruginosa were grown on nutrient-glutamate and harvested during log phase. Half of the cells were treated in the usual way with a sample being applied to a Sephadex G-200 column. The remaining pellet was resuspended in 1 litre of sterile basal medium containing 30 mM-acetate but lacking a supply of nitrogen. The nitrogen-free medium was prepared by omitting the ammonium chloride from the recipe of Ashworth and Kornberg (1966) and replacing it with double distilled water.

The nitrogen-free medium containing a log-phase culture of mutant Ps.aeruginosa was incubated overnight

(16 h) at 37°C. There was no increase in absorbance at 680 nm. Cell growth could not take place in the absence of nitrogen. The cells were harvested and a sample applied to a Sephadex G-200 column. For this experiment to be really meaningful it was essential that all the cells were broken so that a comparison of total citrate synthase activity and protein could be made between the samples.

Fig.20 shows that there was very little change in total protein but there was an increase in citrate synthase activity on nitrogen-free medium containing acetate. This was almost entirely due to an increase in the level of CS II. A similar effect was also seen when other carbon sources were used instead of acetate. Two control experiments were also carried out. Cells from a log-phase culture were transferred to basal medium containing ammonium chloride but lacking a carbon source and to medium lacking both carbon and nitrogen. There was no change in the levels of citrate synthase in both control experiments which showed that any change in the ratio of CS I to CS II on nitrogen-free medium was only brought about in the presence of different carbon compounds.

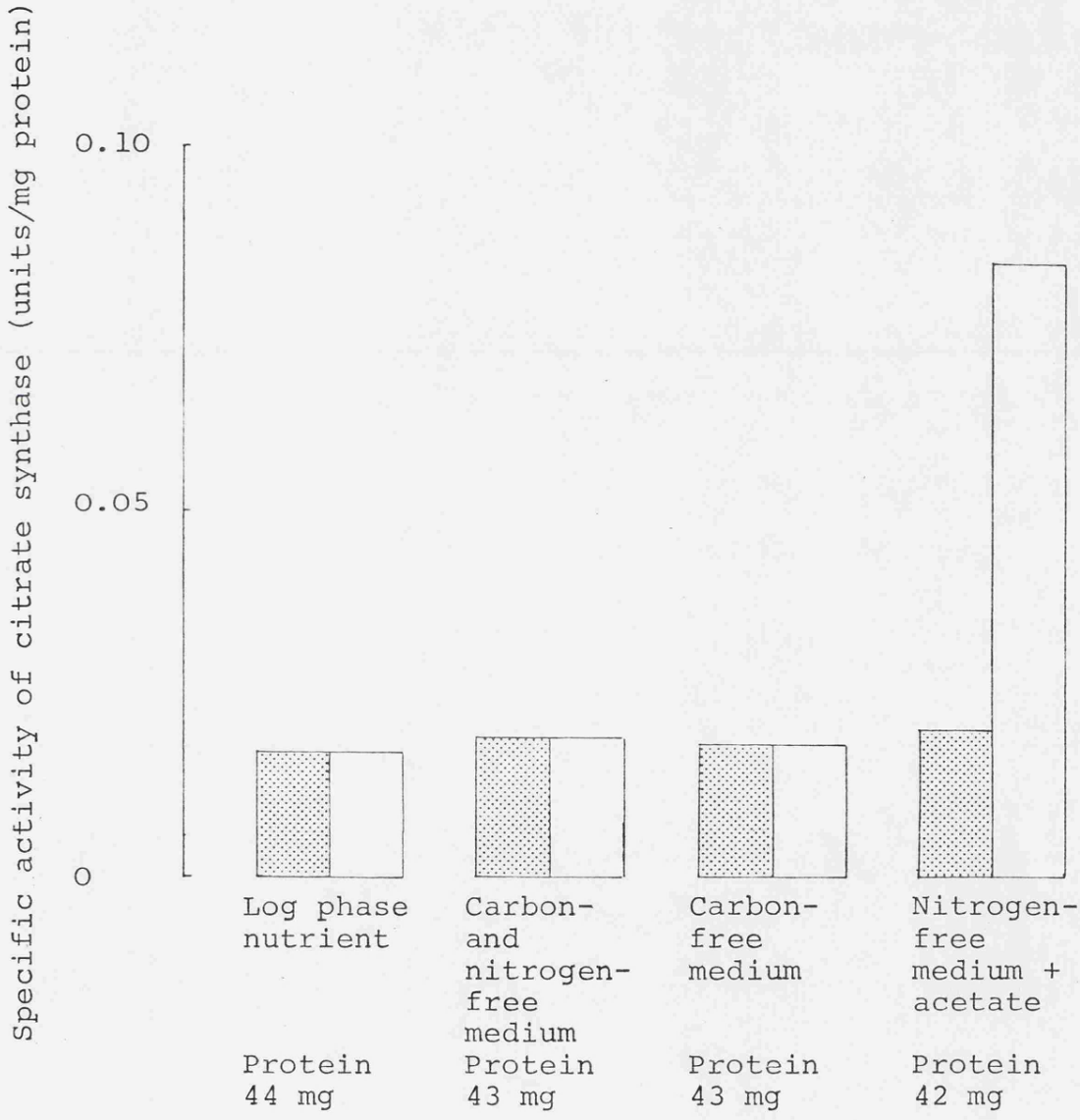


Fig.20. Histogram showing the effect of transfer of a log-phase culture of mutant Ps.aeruginosa to nitrogen-free medium containing 30 mM-acetate on the levels of citrate synthase

▨ CS I, □ CS II.

The experiment was repeated in the presence of chloramphenicol (200 µg/ml) (Fig.21). A log-phase culture was treated with chloramphenicol to stop growth and then transferred to nitrogen-free medium containing acetate and chloramphenicol. The protein synthesis inhibitor prevented an increase in the level of CS II. These experiments indicated that CS II was not a break-down product of CS I but rather a result of de novo protein synthesis.

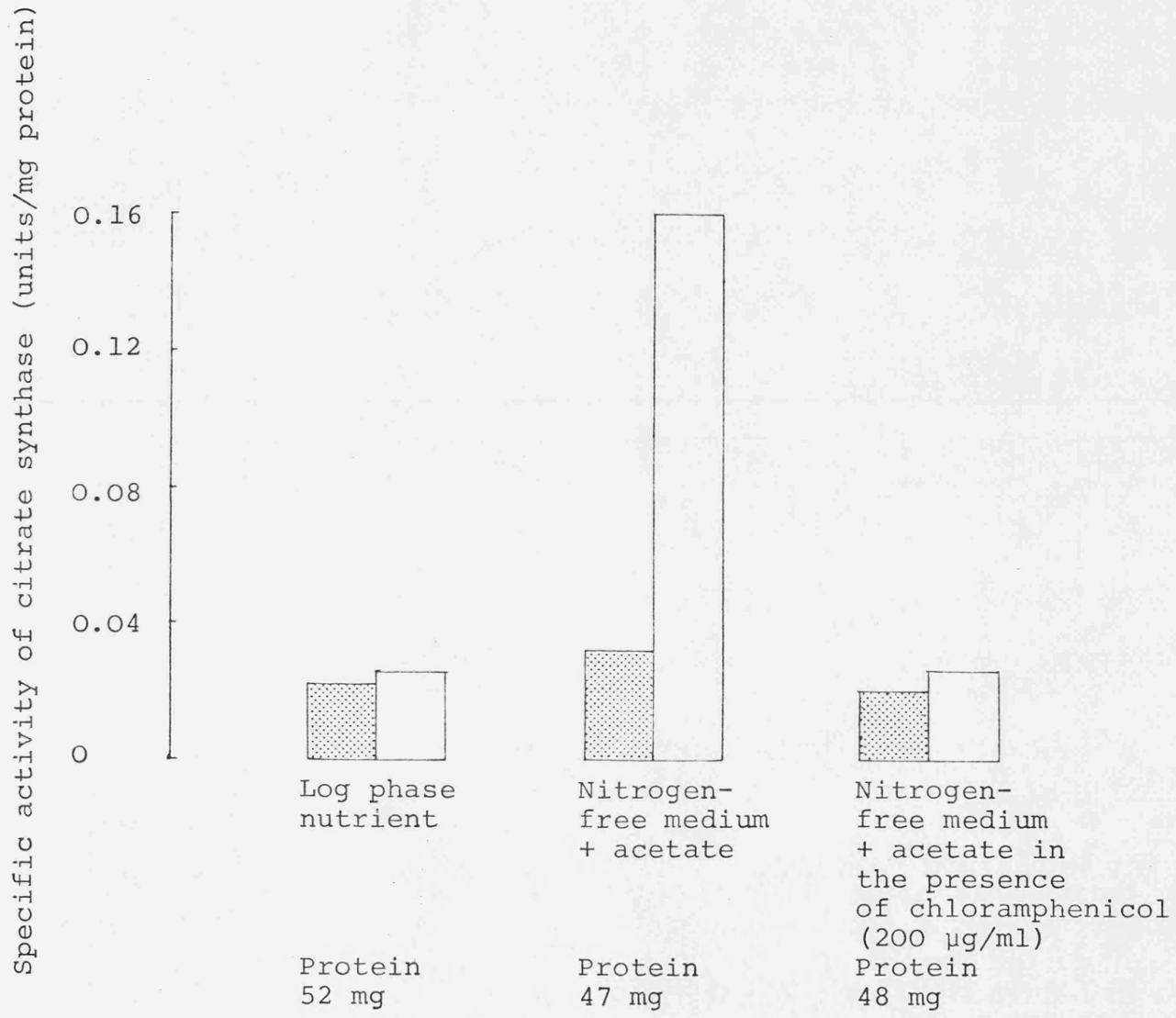


Fig.21. Histogram showing the effect of chloramphenicol on the levels of citrate synthase when a log-phase culture of mutant Ps.aeruginosa is transferred to nitrogen-free medium containing 30 mM-acetate

▒ CS I, □ CS II.

Section B. Purification of Wild-Type and Mutant Ps.aeruginosa Citrate Synthases

A number of bacterial citrate synthases have been purified. The citrate synthase from E.coli has been purified by Weitzman (1969 a), Danson and Weitzman (1973), Tong and Duckworth (1975) and Wright and Sanwal (1971). The enzyme from A.calcoaceticus has been purified by Johnson and Hanson (1974) and more recently by Morse and Duckworth (1980). No purification procedure for Ps.aeruginosa citrate synthase has been published. When the procedure of Danson and Weitzman (1973) was used it was discovered that the wild-type Pseudomonas enzyme became insensitive to NADH. This was probably due to oxidation of thiol groups and this was prevented by lowering the pH of the Tris buffer from 8.0 to 7.0. Altering the pH affected the performance of the chromatography columns and a homogeneous enzyme preparation was not obtained, as shown by polyacrylamide-gel electrophoresis. Insertion of additional steps only resulted in further loss of enzyme activity and no significant purification.

Similarly CS I and CS II from the mutant Ps. aeruginosa were not purified to homogeneity. Enzyme activity was assayed on polyacrylamide disc gels by

slicing the gels into small sections and then adding the assay reagents. For all three enzymes, only one band of activity was detected. Table 7 shows the Rf values of Pseudomonas citrate synthases together with those of citrate synthases from other sources.

Matrex Gel Red A was a very useful additional step in the purification of CS II. Elution of the enzyme was achieved with a mixture of 0.1 mM-CoASH and 0.1 mM-oxaloacetate. This combination did not elute CS I or the wild-type enzyme. Elution of the enzymes was only achieved with 0.2 M-KCl which reduced the specificity of elution.

Use of Immobilized Ligands

This research group has a large number of mutant bacterial citrate synthases none of which has yet been satisfactorily purified. Several of these mutant enzymes are present in very low levels and appear to be very unstable. A purification of revertant type 2 citrate synthase from E.coli (strain AB1623.R6) using the method of Weitzman (1969 a) resulted in less than 3% recovery of activity. Various affinity ligands were therefore prepared in an attempt to improve the recovery and reduce the number of steps involved in the purification procedure.

Table 7

Comparison of Rf values of citrate synthases from different sources on 7% polyacrylamide gels at pH 8.9 and 6.9

Organism	Rf value	
	pH 8.9	pH 6.9
Wild-type *Ps.aeruginosa*	0.244	0.165
Mutant *Ps.aeruginosa* CS I	0.242	0.162
A.calcoaceticus	0.264	0.191
Pig Heart	0.513	0.219
Mutant *Ps.aeruginosa* CS II	0.597	0.312
B.megaterium	0.694	0.435

Affinity chromatography exploits the unique biological specificity of the protein-ligand interaction. Acetyl-CoA is a substrate of citrate synthase and it was considered a potential ligand for the affinity chromatography of this enzyme. However, the search for methods for the covalent attachment of nicotinamide nucleotides to insoluble matrices has been hindered by their lack of reactivity (Dean and Lowe, 1972). The 6-amino group of the adenine residue appeared to be a good point of attachment.

CoASH was attached directly to Sepharose (Procedure 1) and also to a 6-carbon spacer (Procedure 2) and then acetylated. Pure pig heart citrate synthase bound to both ligands, but there was only 30% recovery with 0.2 M-KCl. Similar results were also obtained with revertant type 2 citrate synthase. The insertion of a spacer to eliminate steric hindrance did not appear to alter the protein-ligand binding process.

These two methods indicated that there was a very powerful binding between the ligand and enzyme or perhaps the ligand was not attached through the amino group. Barry and O'Carra (1973) considered the 6-amino position of the adenine residue to be a desirable point for immobilization but found it to be so inert that it was

not possible to attach it to the matrix; they suggested that binding was more likely to occur through the OH group of the ribose. It was also possible that the effects observed were ion-exchange phenomena resulting from the introduction of charged groups by the chemical procedures employed (Lowe and Dean, 1974).

There was no difference in results when Activated CH-Sepharose (Procedure 3) was used even though this compound was supposed to react specifically with amino groups, whereas, with the other two methods, the precise mode of action was not absolutely clear. The advantage of Procedure 3 was that acetyl-CoA could be coupled directly without any additional reagents. Similar amounts of acetyl-CoA were found to be immobilized to Sepharose using the three different methods (Table 8).

Dephospho-CoASH was coupled through the adjacent OH groups on the 2'-3' position of the ribose (Procedure 6) and then acetylated. There was no retention of citrate synthase, indicating the importance of the phosphate, or possibly the steric orientation, in enzymic recognition of acetyl-CoA. This was unfortunate because this method was by far the most efficient way of coupling the ligand (3.55 μmoles bound/ml Sepharose).

Table 8

The binding of acetyl-CoA to various Sepharose derivatives

Procedure	Coupling groups available µmoles/ml Sepharose	µmoles bound/ml Sepharose
1. CNBr-Sepharose	unknown	1.50
2. CH-Sepharose	12	1.75
3. Activated CH-Sepharose	12	1.82
4. Activated Thiol-Sepharose	1	0.85*

*CoASH bound

Procedure 4 enabled CoASH to be bound to the matrix through the thiol group, but again there was no retention of enzyme. This illustrated how important was the acetyl group region in enzymic recognition. 3',5'-ADP can be considered as a 'half-molecule' of CoASH and it has been shown by Barry et al. (1976) that it can be used to purify the CoASH-dependent enzyme succinyl-CoA synthetase when bound as the N^6-(6-aminohexyl) 3',5'-ADP analogue to Sepharose. This ligand was tested with citrate synthase.

The ligand was prepared by reacting Activated CH-Sepharose with 3',5'-ADP. No binding of citrate synthase from Ps.aeruginosa or pig heart was achieved. The results with acetyl-CoA and its analogues indicated that this ligand had no potential for the purification of citrate synthase.

It was decided to investigate the possibility of immobilizing the other substrate of citrate synthase, oxaloacetate, as well as various other citric acid cycle intermediates (α-oxoglutarate, citrate and isocitrate). These compounds were bound to the matrix through their carboxyl groups using AH-Sepharose (Procedure 5). Pure pig heart citrate synthase bound to citrate-Sepharose and a 3-fold recovery of activity was obtained with

0.2 M-KCl. It was known that this concentration of salt stimulated pig heart citrate synthase. However with revertant type 2, only 30% recovery was obtained with a 5-fold purification. Earlier work had shown KCl to inhibit the enzyme.

In order to check the possibility that these results were due to ion-exchange rather than affinity chromatography, citrate synthase was applied to Sepharose, AH-Sepharose and CH-Sepharose under the same conditions. There was no retention of enzyme on Sepharose or CH-Sepharose but the elution profile from AH-Sepharose was identical with that obtained from citrate-Sepharose confirming ion-exchange properties. A sensitive method for the detection and differentiation of COOH and NH_2 groups was required. Titration curves showed that a difference between these groups could be detected. Acid-base titrations of α-oxoglutarate-, oxaloacetate- and isocitrate-Sepharoses suggested the presence of both COOH and NH_2 groups. Thus this procedure was of no potential value until all the coupling groups had been fully substituted.

As well as immobilizing substrates as ligands in affinity chromatography, effectors can also be used.

AMP, ADP, ATP and NADH were bound to Sepharose-hydrazide through the adjacent OH groups on the 2'-3' position of the ribose using the periodate-oxidation method (Procedure 6). These ligands would not be expected to be as specific as the substrates because they are involved in a large number of other enzymic reactions.

Pure pig heart citrate synthase was found to bind to ATP-Sepharose and was recovered by elution with 1 mM-ATP (Fig.22). A much sharper elution was obtained with a combination of 0.1 mM-CoASH and 0.1 mM-oxaloacetate. This provided an extra degree of specificity. Only 20% of the enzyme bound to AMP-Sepharose and it was more difficult to recover. These results were consistent with the effectiveness of inhibition of activity by adenine nucleotides (ATP>ADP>AMP) exhibited by citrate synthases from eukaryotes and Gram-positive bacteria.

Revertant type 2 citrate synthase bound to AMP-, ADP- and ATP-Sepharoses but, at best, only 20% recovery could be obtained. Revertant type 3 citrate synthase from E.coli (strain AB1623.R9) bound to these ligands and 50% recovery with a 25-fold purification was obtained by elution with 0.1 mM-CoASH and 0.1 mM-oxaloacetate from ATP-Sepharose. None of the citrate synthases from

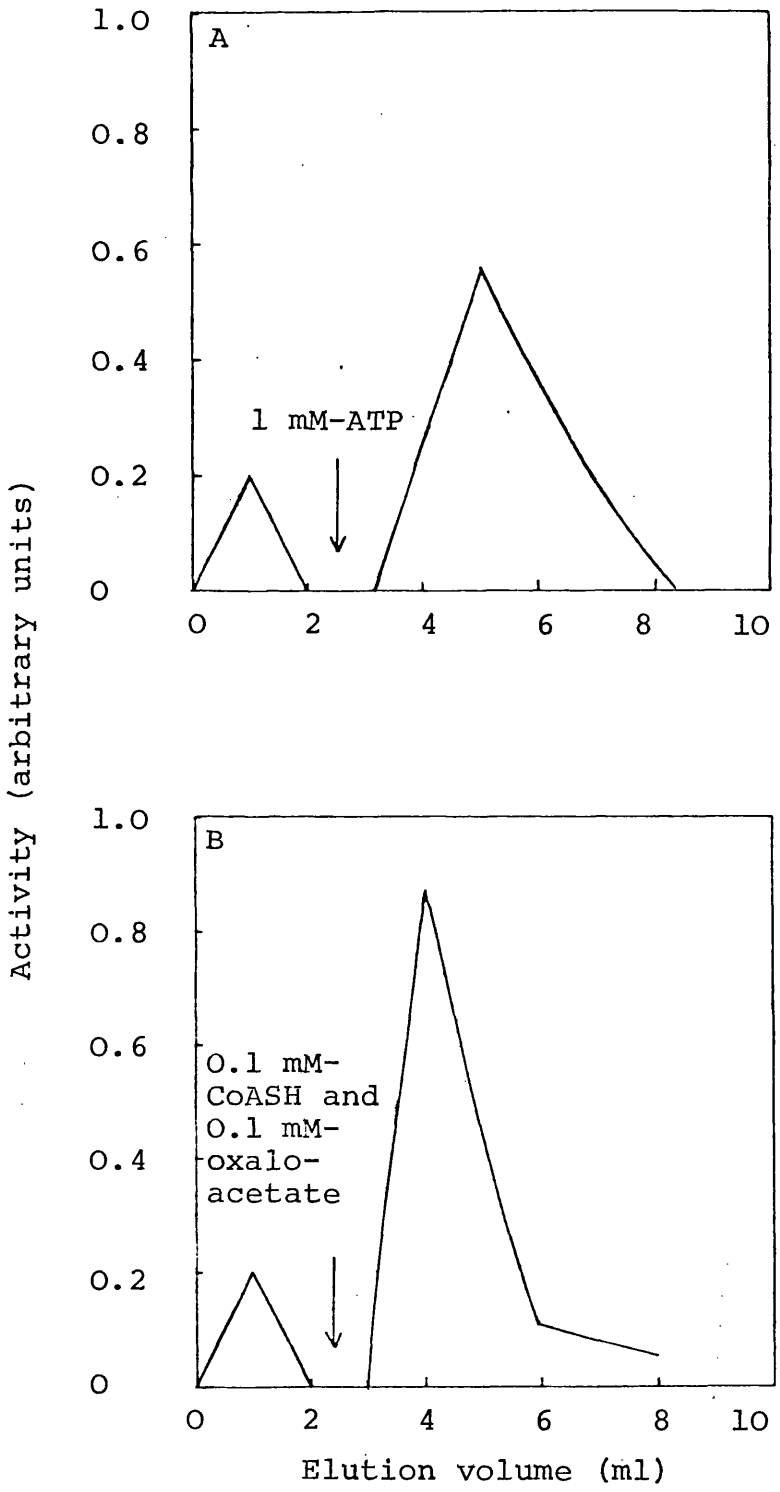


Fig.22. Affinity chromatography of pig heart citrate synthase on ATP-Sepharose
The sample (0.1 mg, 0.2 ml) was applied and eluted with 'Tris buffer (pH 8.0)'. At the point shown by the arrows the eluent was changed to:
A 1 mM-ATP in 'Tris buffer (pH 8.0)',
B 0.1 mM-CoASH and 0.1 mM-oxaloacetate in 'Tris buffer (pH 8.0)'.

wild-type and mutant Ps.aeruginosa bound to these ligands. Chromatography on ATP-Sepharose has been shown to be a very useful step in the purification of eukaryotic citrate synthases (Mukherjee and Srere, 1976).

No binding of enzyme was observed to commercially available 5'-AMP-Sepharose, in which the nucleotide was attached through the 6-amino group. The point of attachment of adenine nucleotides appeared to be important for enzyme recognition of ligands.

NADH is an effector of wild-type E.coli citrate synthase. When a crude extract of this enzyme was applied to NADH-Sepharose, there was no binding of citrate synthase but 85% of the malate dehydrogenase became bound. However, the enzyme could not be eluted. Pure malate dehydrogenase was applied to a Sepharose-adipic acid dihydrazide conjugate and similar results were obtained. This was contrary to the results of Lamed et al. (1973) who claimed that unreacted hydrazide groups had no ion-exchange properties on the resin at neutral or high pH.

Thus it was possible to immobilize substrates and effectors of citrate synthase but, apart from ATP-Sepharose,

the immobilized ligands appear to be of little value for the purification of citrate synthase. It may well be that the use of immobilized group-selective dyes will be more successful in the purification of this enzyme.

Succinyl-CoA synthetase and NADP-linked isocitrate dehydrogenase are two other enzymes in which this research group is interested. The periodate-oxidation method of attachment of nucleotides through adjacent OH groups on the 2'-3' position of the ribose residue has been applied to the affinity chromatography of both of these enzymes with some success.

Pure pig heart NADP-linked isocitrate dehydrogenase was applied to $NADP^+$-Sepharose and 50% recovery of enzyme activity was obtained by elution with 1 mM-$NADP^+$. This method has been used previously by Hy and Reeves (1976) to purify isocitrate dehydrogenase from E.coli.

A.calcoaceticus has two NADP-linked isocitrate dehydrogenases, one of which is stimulated by AMP (Parker and Weitzman, 1970; Self and Weitzman, 1972). It may be possible to bind both of these enzymes to $NADP^+$-Sepharose and then elute them selectively.

Succinyl-CoA synthetase from A.calcoaceticus requires GDP as a substrate whereas the E.coli enzyme requires ADP. The A.calcoaceticus enzyme has been applied to GDP-Sepharose, 100% recovery and a 30-fold purification being obtained by elution with 1 mM-GDP. The elution with succinyl-CoA was less efficient. This method has been used in the purification of succinyl-CoA synthetase from rat liver (Ball and Nishimura, 1980). E.coli succinyl-CoA synthetase has been applied to ADP-Sepharose and eluted with 1 mM-ADP. These ligands thus seem to have potential for the purification of diverse succinyl-CoA synthetases.

Section C. Characterization of Citrate Synthases from Wild-Type and Mutant Ps.aeruginosa

A comparative study of some of the properties of the citrate synthase found in wild-type Ps.aeruginosa and the two enzymes CS I and CS II found in the mutant Ps.aeruginosa has been made. The properties investigated were molecular weight, Km values for both substrates and the effect of various nucleotides on activity. In addition, the effects of pH, heat, various thiol-blocking reagents and photo-oxidation were also investigated.

Molecular Weight

The molecular weights of citrate synthases from wild-type and mutant Ps.aeruginosa were determined by gel filtration on Sephadex G-200 in the presence of marker proteins of known molecular weight according to the method of Andrews (1965). Fig.23 shows the relationship between Stokes' radius and elution volume from the column. The citrate synthase from the wild-type organism and CS I both had a molecular weight of about 300,000 whereas CS II had a molecular weight of about 100,000. These were similar to the values determined by Massarini and Cazzulo (1975) for the two forms of citrate synthase in a marine pseudomonad.

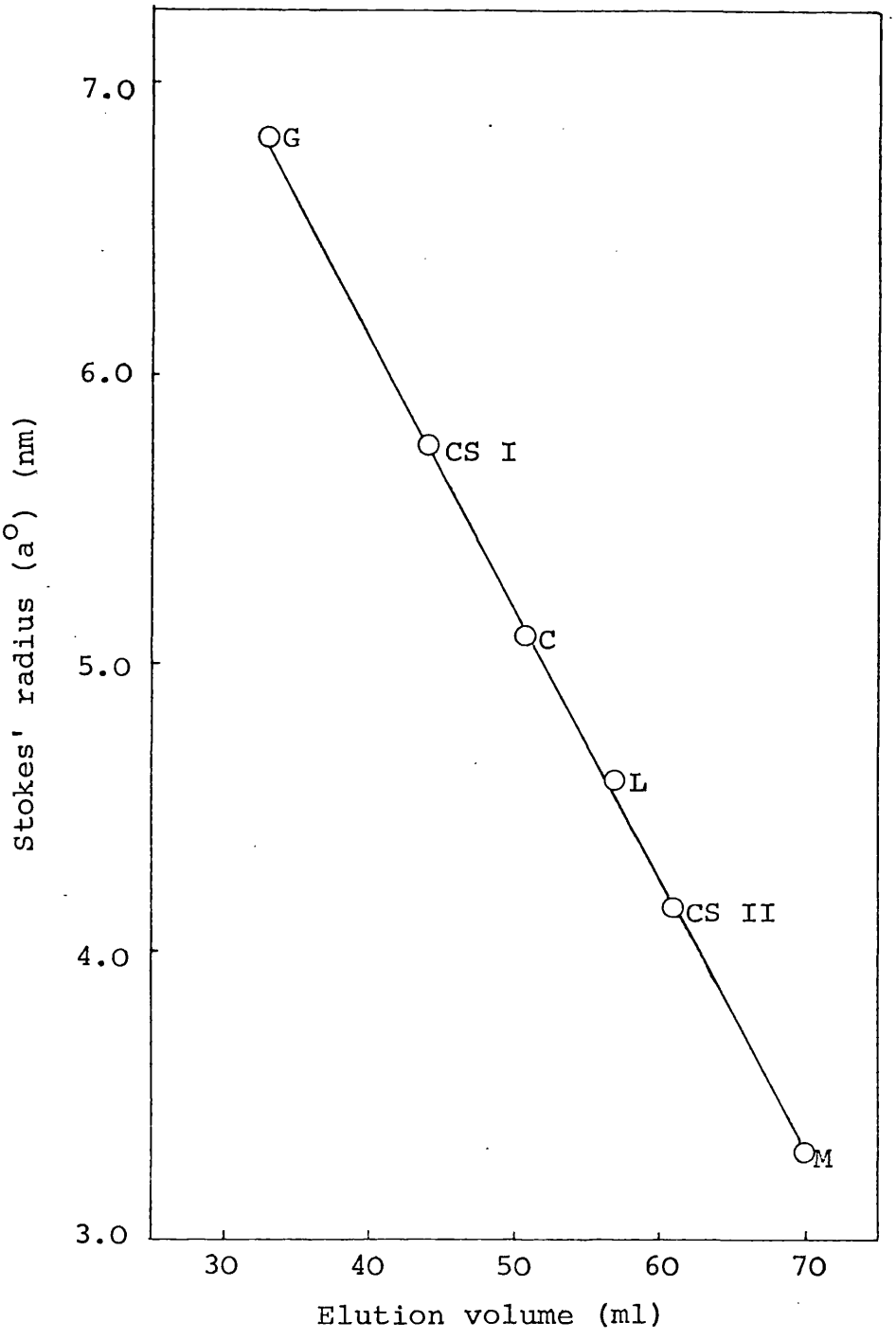


Fig.23. Relationship between the Stokes' radii of proteins and their comparative elution volumes from a Sephadex G-200 gel filtration column (35.0 x 2.5 cm) The proteins used were β-galactosidase (G), 'large' citrate synthase from wild-type and mutant Ps. aeruginosa (CS I), catalase (C), 'small' citrate synthase from mutant Ps.aeruginosa (CS II) and malate dehydrogenase (M).

These were only estimations and a more accurate determination using analytical ultracentrifugation is required. However, they did show that CS I was about three times the weight of CS II and that CS I was very similar, if not identical, to the wild-type citrate synthase.

Kinetic and Regulatory Properties

The dependence of enzyme activity on substrate concentrations was determined and the apparent Km values for acetyl-CoA and oxaloacetate calculated. Hill coefficients were also calculated.

Double reciprocal plots showed a sigmoidal dependence of rate on acetyl-CoA in the wild-type citrate synthase which became hyperbolic in the presence of 0.1 M-KCl (Fig.24). The cooperativity observed in the absence of KCl was also indicated by Hill plots where the Hill coefficient (n) was 1.98 whereas, in the presence of KCl, the value of n was 0.95 (Fig.25). With oxaloacetate as the variable substrate the dependence of enzyme activity was hyperbolic (Fig.26) and the Hill coefficient was 1.03 (Fig.27).

CS I showed a sigmoidal dependence of rate on both substrates and this was also reflected in the values of

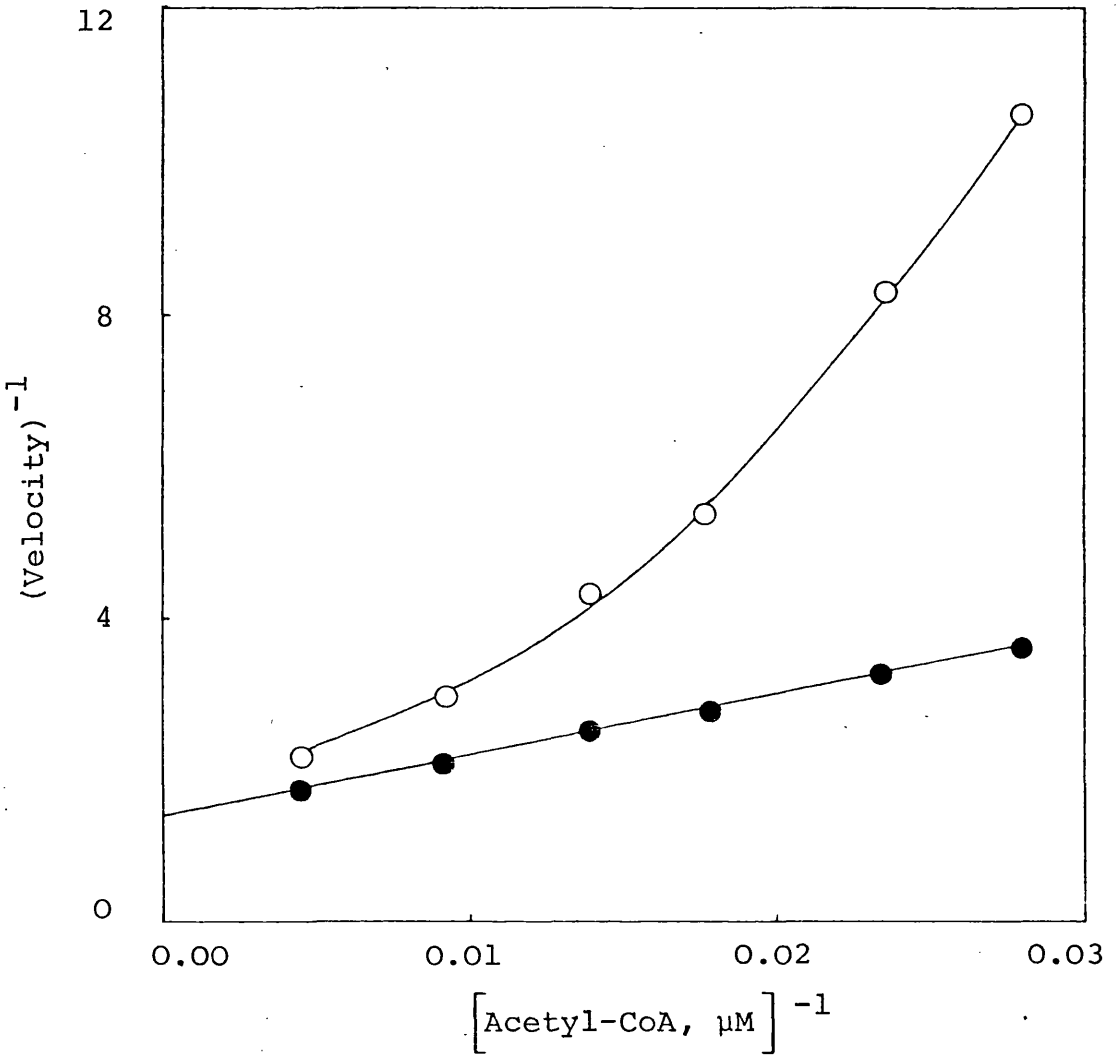


Fig.24. Double reciprocal plots of the rate dependence of wild-type Ps.aeruginosa citrate synthase activity on the concentration of acetyl-CoA
Enzyme activity (in arbitrary units) was measured using assay Method 1 at a fixed oxaloacetate concentration of 0.16 mM.
In 'Tris buffer (pH 8.0)': (○) in the absence of KCl;
(●) in the presence of KCl.

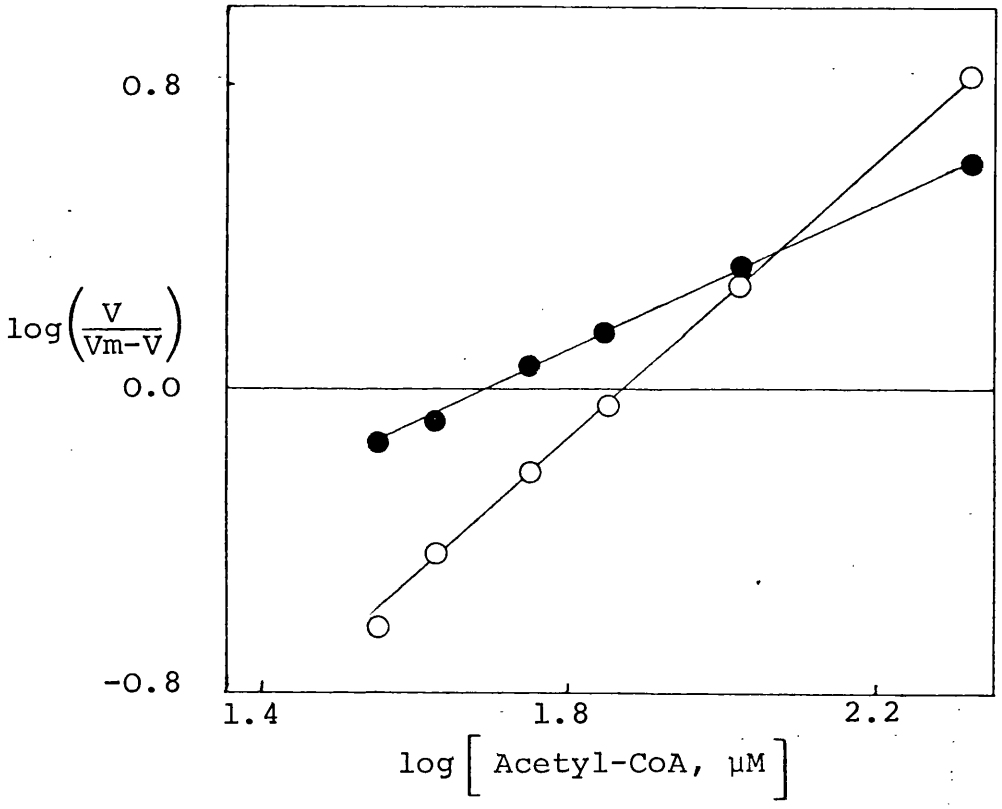


Fig.25. Hill plots of the rate dependence of wild-type Ps.aeruginosa citrate synthase activity on the concentration of acetyl-CoA
Data were taken from Fig.24.
(○) In 'Tris buffer (pH 8.0)' in the absence of KCl;
(●) In 'Tris buffer (pH 8.0)' in the presence of KCl.

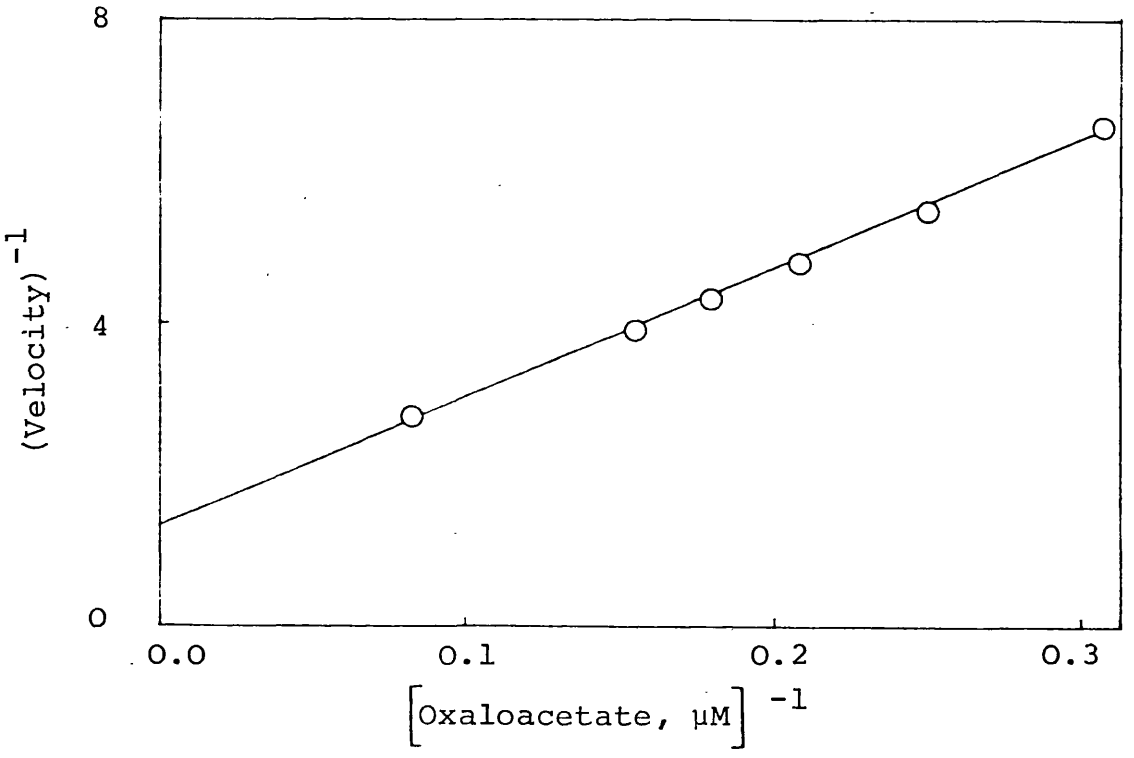


Fig.26. Double reciprocal plot of the rate dependence of wild-type Ps.aeruginosa citrate synthase activity on the concentration of oxaloacetate
Enzyme activity (in arbitrary units) was measured using assay Method 1 at a fixed acetyl-CoA concentration of 0.14 mM. In 'Tris buffer (pH 8.0)'.

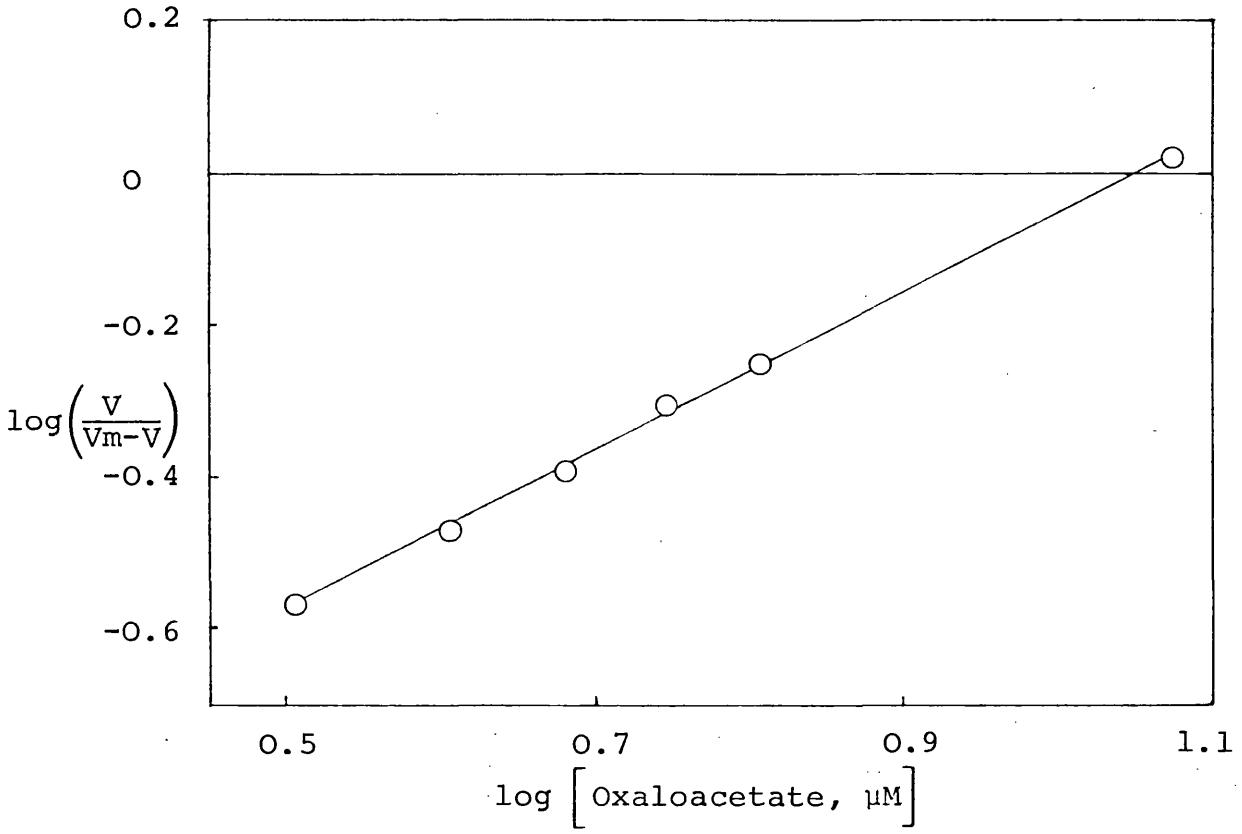


Fig.27. Hill plot of the rate dependence of wild-type Ps.aeruginosa citrate synthase activity on the concentration of oxaloacetate
Data were taken from Fig.26.
In 'Tris buffer (pH 8.0)'.

the Hill coefficients (Figs.28-31). The $S_{0.5}$ values for both substrates were considerably greater than those for the wild-type enzyme (Table 9). In fact, the $S_{0.5}$ values were so large that, under standard assay conditions, the concentration of fixed substrate (either 0.14 mM-acetyl-CoA or 0.16 mM-oxaloacetate) was much lower than the determined $S_{0.5}$ value for that substrate. The $S_{0.5}$ values were therefore also determined in the presence of a ten-fold increase in the concentration of fixed substrate (either 1.40 mM-acetyl-CoA or 1.60 mM-oxaloacetate). This resulted in a lowering of the $S_{0.5}$ values for both substrates as well as the Hill coefficients (Table 9), but they were still greater than those found in the wild-type enzyme. The sigmoidicity was not altered substantially in the presence of 0.1 M-KCl.

CS II showed a hyperbolic dependence of rate on both substrates (Figs.32-35) and the apparent Km values were similar to those of the 'large' wild-type enzyme.

Table 10 shows the effect of various nucleotides on the activity of wild-type and mutant Ps.aeruginosa citrate synthases. These effects were similar to those described by Weitzman and Danson (1976) for 'large' and 'small' citrate synthases. There was one exception, which was the activation of the enzyme by AMP

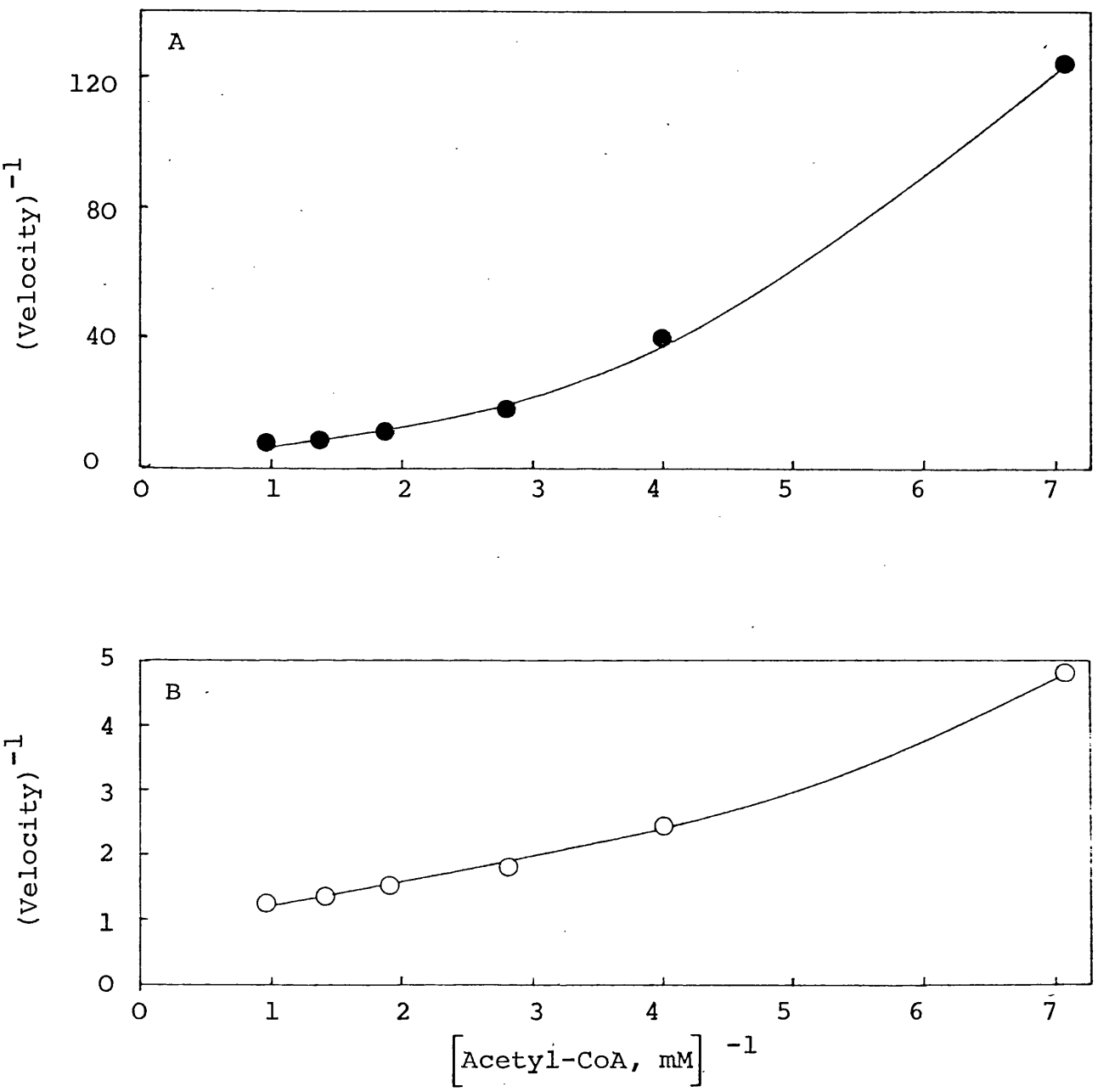


Fig.28. Double reciprocal plots of the rate dependence of mutant Ps.aeruginosa CS I activity on the concentration of acetyl-CoA

Enzyme activity (in arbitrary units) was measured using assay Method 1 at a fixed oxaloacetate concentration.

In 'Tris buffer (pH 8.0)': A (●) 0.16 mM-oxaloacetate;
B (○) 1.60 mM-oxaloacetate.

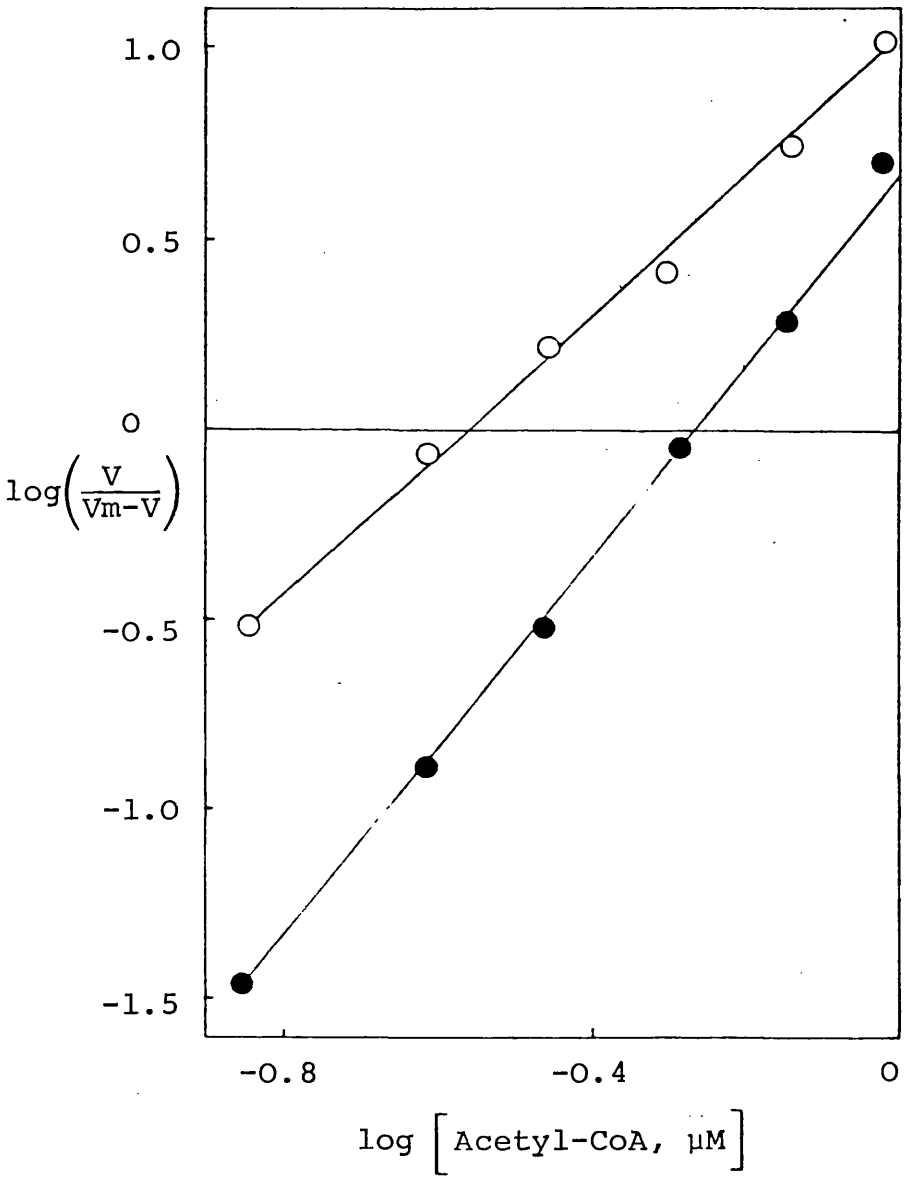


Fig.29. Hill plots of the rate dependence of mutant Ps.aeruginosa CS I activity on the concentration of acetyl-CoA

Data were taken from Fig.28.

(●) In 'Tris buffer (pH 8.0)' at 0.16 mM-oxaloacetate;

(○) In 'Tris buffer (pH 8.0)' at 1.60 mM-oxaloacetate.

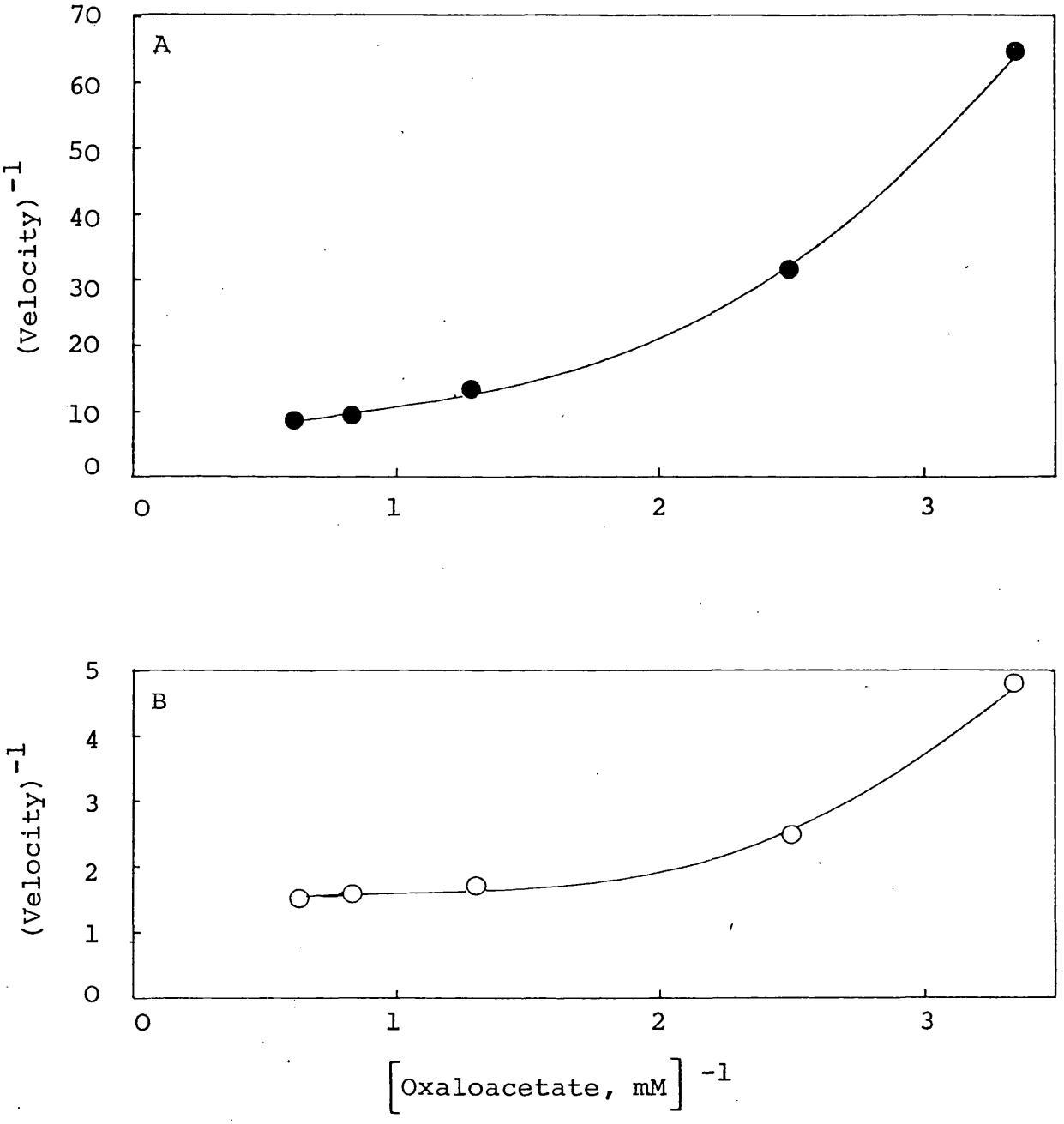


Fig.30. Double reciprocal plots of the rate dependence of mutant Ps.aeruginosa CS I activity on the concentration of oxaloacetate

Enzyme activity (in arbitrary units) was measured using assay Method 1 at a fixed acetyl-CoA concentration.

In 'Tris buffer (pH 8.0)': A(●)0.16 mM-acetyl-CoA;
B(○)0.60 mM-acetyl-CoA.

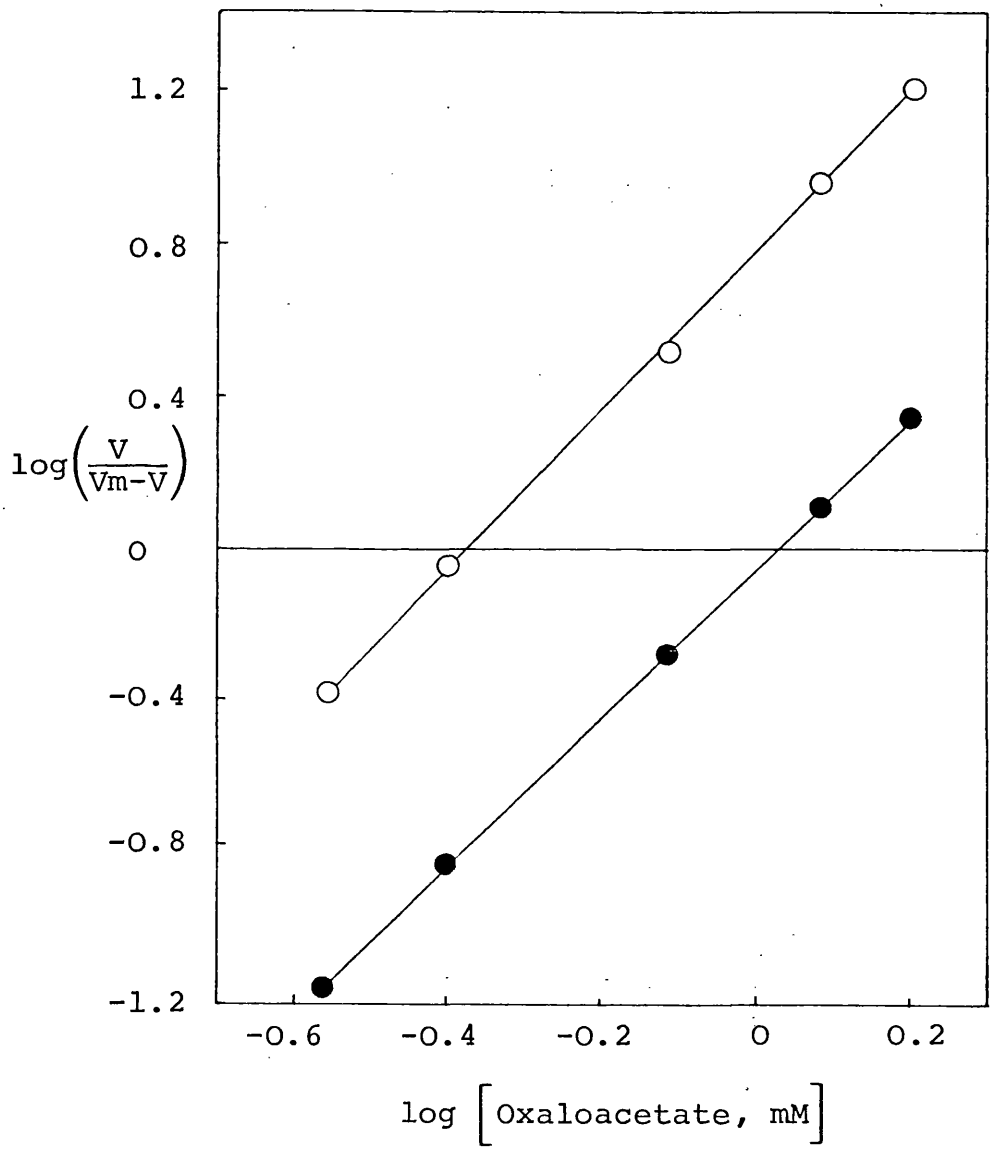


Fig.31. Hill plots of the rate dependence of mutant Ps.aeruginosa CS I activity on the concentration of oxaloacetate
Data were taken from Fig.30.
(●) In 'Tris buffer (pH 8.0)' at 0.14 mM-acetyl-CoA;
(○) In 'Tris buffer (pH 8.0)' at 1.40 mM-acetyl-CoA.

Table 9

Kinetic parameters of citrate synthases from wild-type and mutant Ps.aeruginosa

Enzyme	Oxaloacetate as variable substrate (at 0.14 mM-acetyl-CoA)		Acetyl-CoA as variable substrate (at 0.16 mM-oxaloacetate)	
	Apparent Km or $S_{0.5}$ (μM)	Hill coefficient	Apparent Km or $S_{0.5}$ (μM)	Hill coefficient
Wild-type citrate synthase	11.8 ± 1.0	1.03	76.9 ± 3.3	1.98
Mutant CS I	677 ± 79	2.10	704 ± 80	3.50
	370 ± 20*	2.00	266 ± 15†	1.75
Mutant CS II	10.3 ± 0.8	0.98	51.3 ± 3.8	1.00

* at 1.40 mM-acetyl-CoA

† at 1.60 mM-oxaloacetate

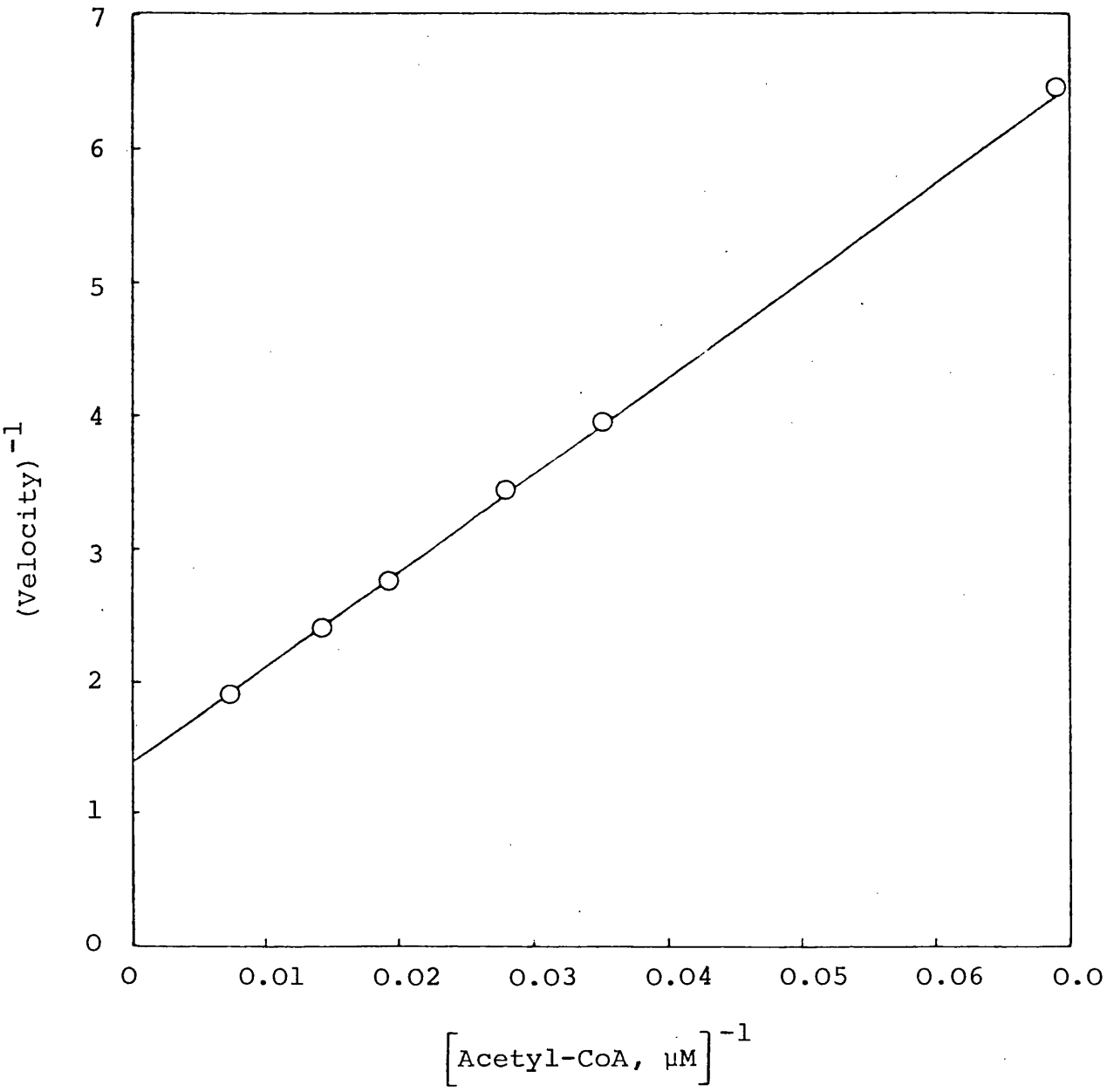


Fig.32. Double reciprocal plot of the rate dependence of mutant Ps.aeruginosa CS II activity on the concentration of acetyl-CoA
Enzyme activity (in arbitrary units) was measured using assay Method 1 at a fixed oxaloacetate concentration of 0.16 mM.
In 'Tris buffer (pH 8.0)'.

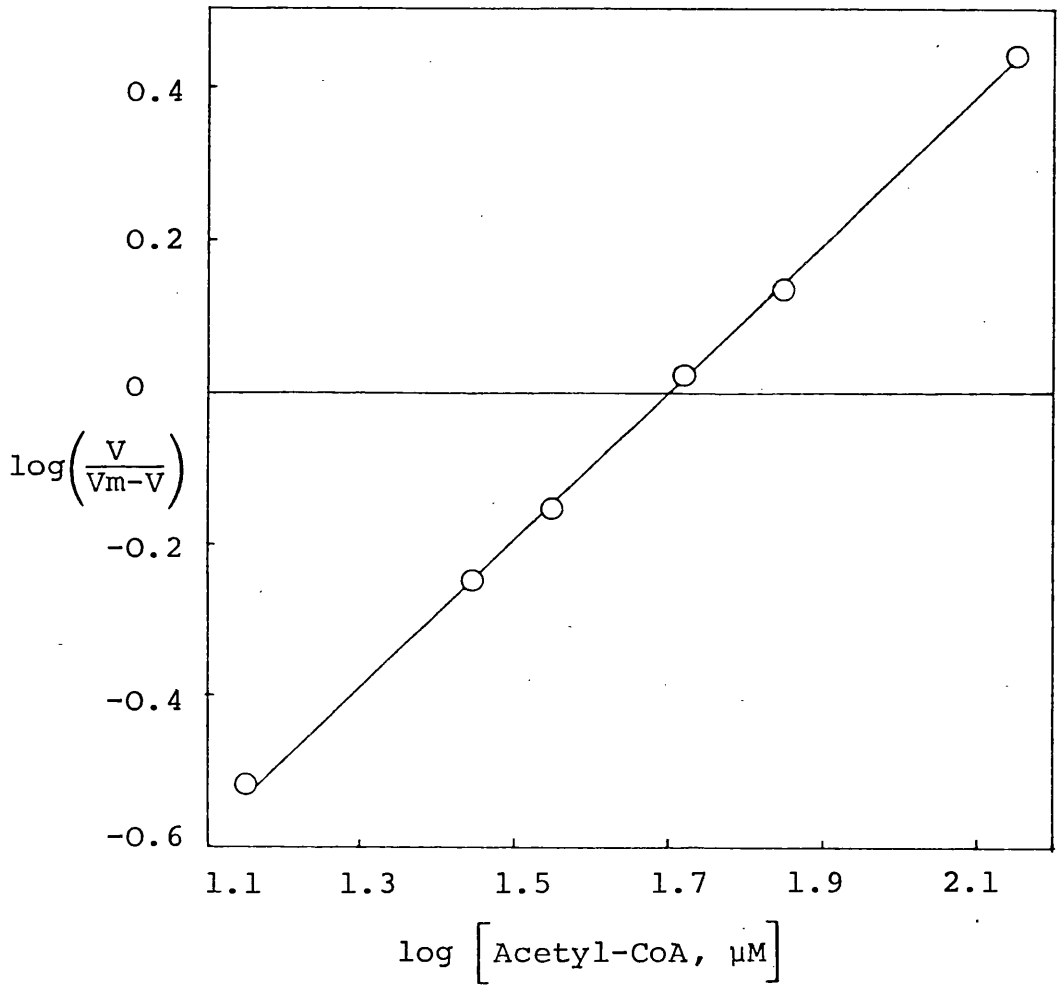


Fig.33. Hill plot of the rate dependence of mutant Ps.aeruginosa CS II activity on the concentration of acetyl-CoA
Data were taken from Fig.32.

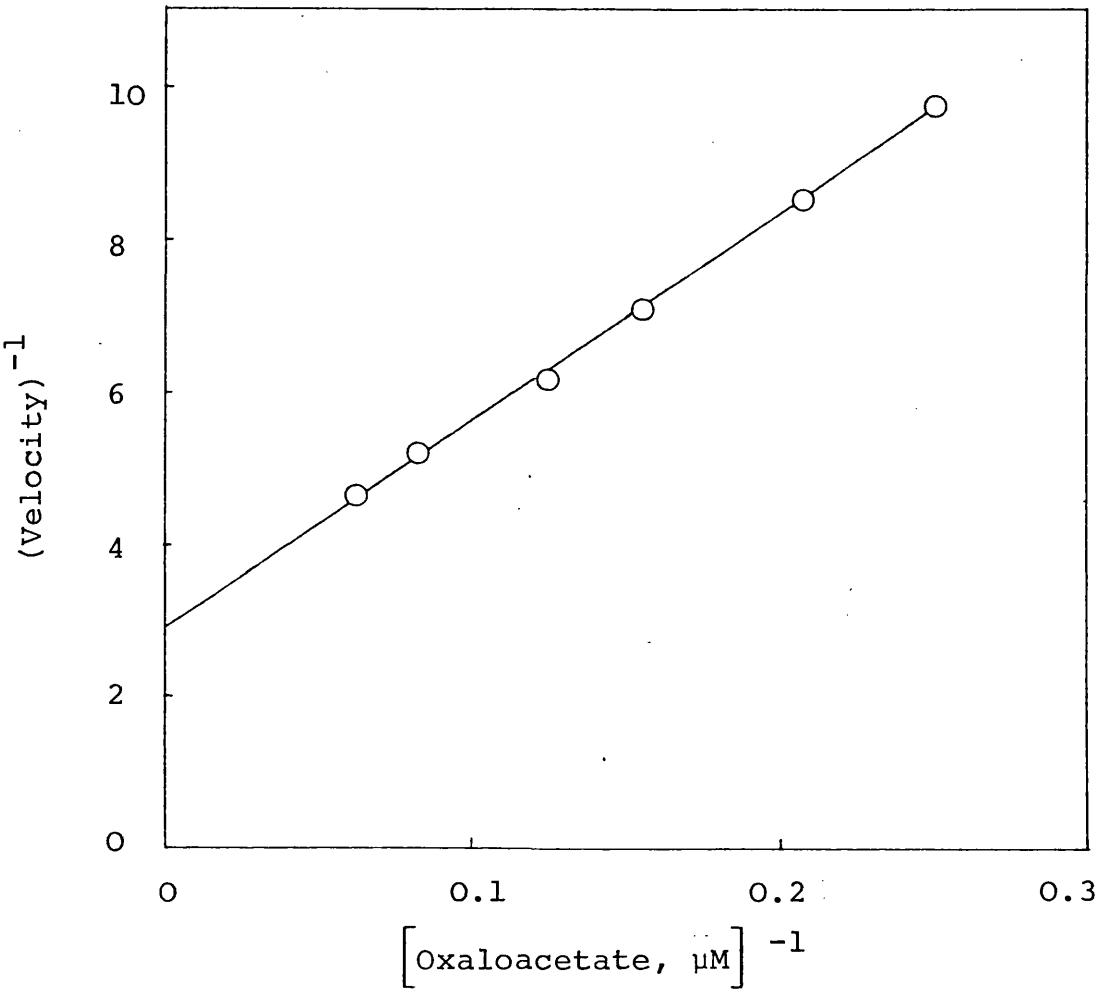


Fig.34. Double reciprocal plot of the rate dependence of mutant Ps.aeruginosa CS II activity on the concentration of oxaloacetate
Enzyme activity (in arbitrary units) was measured using assay Method 1 at a fixed acetyl-CoA concentration of 0.14 mM.
In 'Tris buffer (pH 8.0)'.

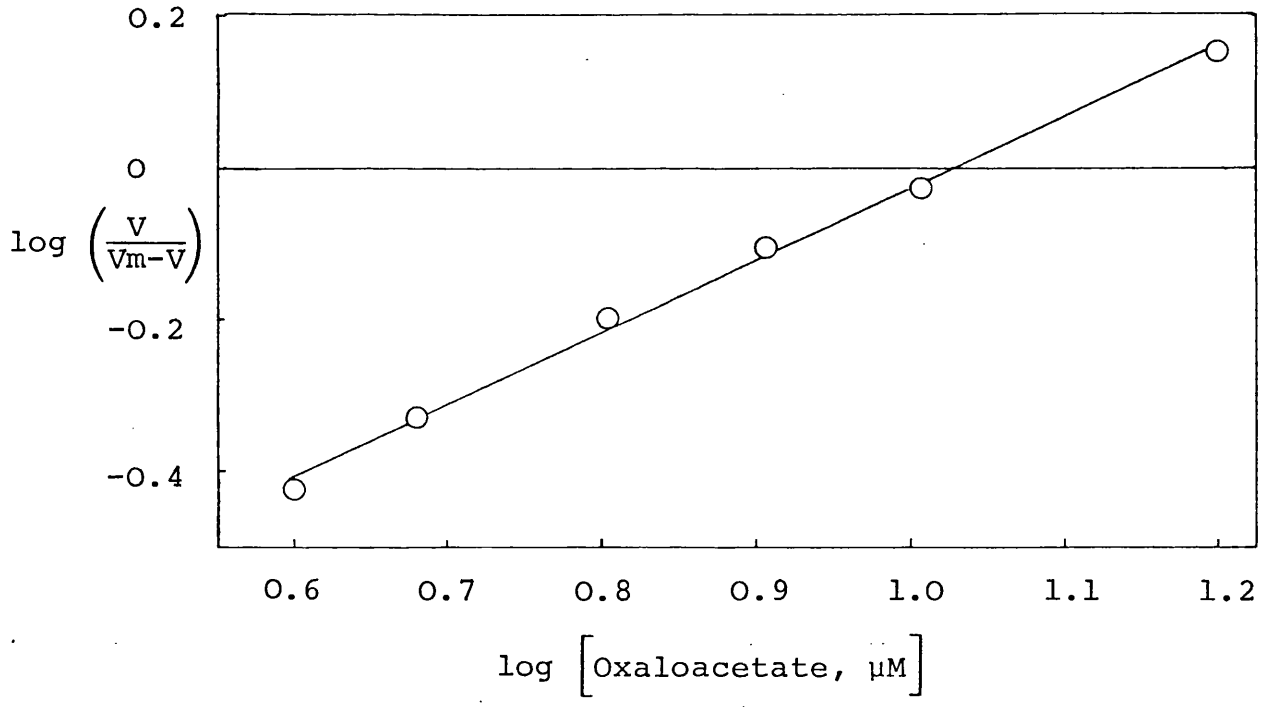


Fig.35. Hill plot of the rate dependence of mutant Ps.aeruginosa CS II activity on the concentration of oxaloacetate
Data were taken from Fig.34.

Table 10

Nucleotide inhibition of wild-type and mutant Ps.aeruginosa citrate synthases

The inhibitions were determined at an acetyl-CoA concentration of 0.14 mM.

Enzyme	Concentration (mM)	Nucleotide inhibition (%)*						
		ATP	ADP	AMP	NADPH	NADH	$NADP^+$	NAD^+
Wild-type	0.5	12	4	0	4	72	4	4
citrate	1.0	20	12	-3	16	72	6	8
synthase	2.0	37	20	-7	20	72	9	12
Mutant	0.5	5	-40	-700	25	63	6	16
CS I	1.0	12	-98	-1275	35	72	11	24
	2.0	26	-140	-1500	47	75	20	34
Mutant	0.5	12	0	-12	0	0	3	0
CS II	1.0	24	4	-17	6	5	7	5
	2.0	44	5	-20	8	8	10	10

*-ve values of inhibitions correspond to activation effects

and this was most marked in CS I (Fig.36). Activation by phosphate ion was also observed, but this was probably due to a non-specific ionic effect as it was also produced by other salts at equivalent ionic strength.

'Large' citrate synthases are sensitive to inhibition by NADH which is an end product of the citric acid cycle. In addition, in strict aerobes, this inhibition is overcome by AMP (Weitzman and Jones, 1968).

Ps.aeruginosa, being a strict aerobe, provided the opportunity to investigate the effect of both NADH and AMP on citrate synthase activity. Fig.37 shows the effect of NADH on the activity of wild-type enzyme and mutant CS I. The wild-type enzyme produced a sigmoidal dependence of inhibition on NADH concentration which was more apparent in the presence of 0.1 M-KCl. The enzyme was desensitized to NADH inhibition by treatment with the thiol-blocking reagent DTNB. A slight inhibition was still observed, but this was due to a non-specific isosteric nucleotide inhibition as produced by other nucleotides - NADPH, $NADP^+$ and NAD^+ (Table 10). The enzyme lost its sensitivity to NADH inhibition if kept in 'Tris buffer (pH 8.0)' for longer than 24 h. This

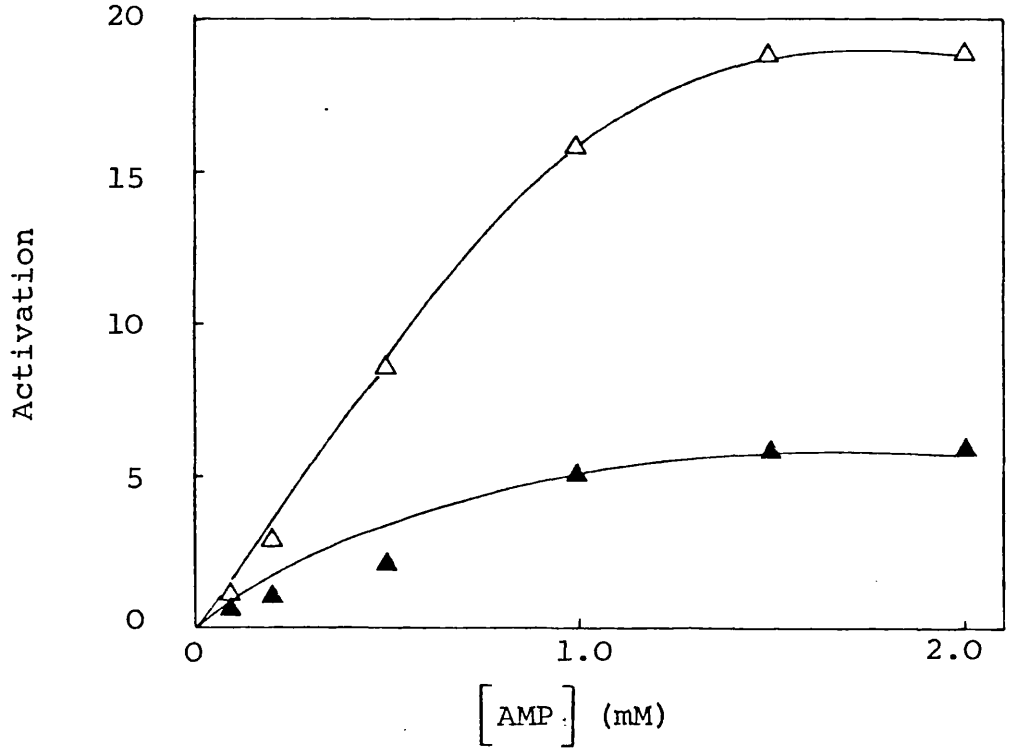


Fig.36. Activation of mutant Ps.aeruginosa CS I by AMP

Enzyme activity was measured using assay Method 1 at a fixed acetyl-CoA concentration of 0.14 mM and an oxaloacetate concentration of 0.16 mM. In 'Tris buffer (pH 8.0)':(△) In the absence of KCl; (▲) In the presence of 0.1 M-KCl.

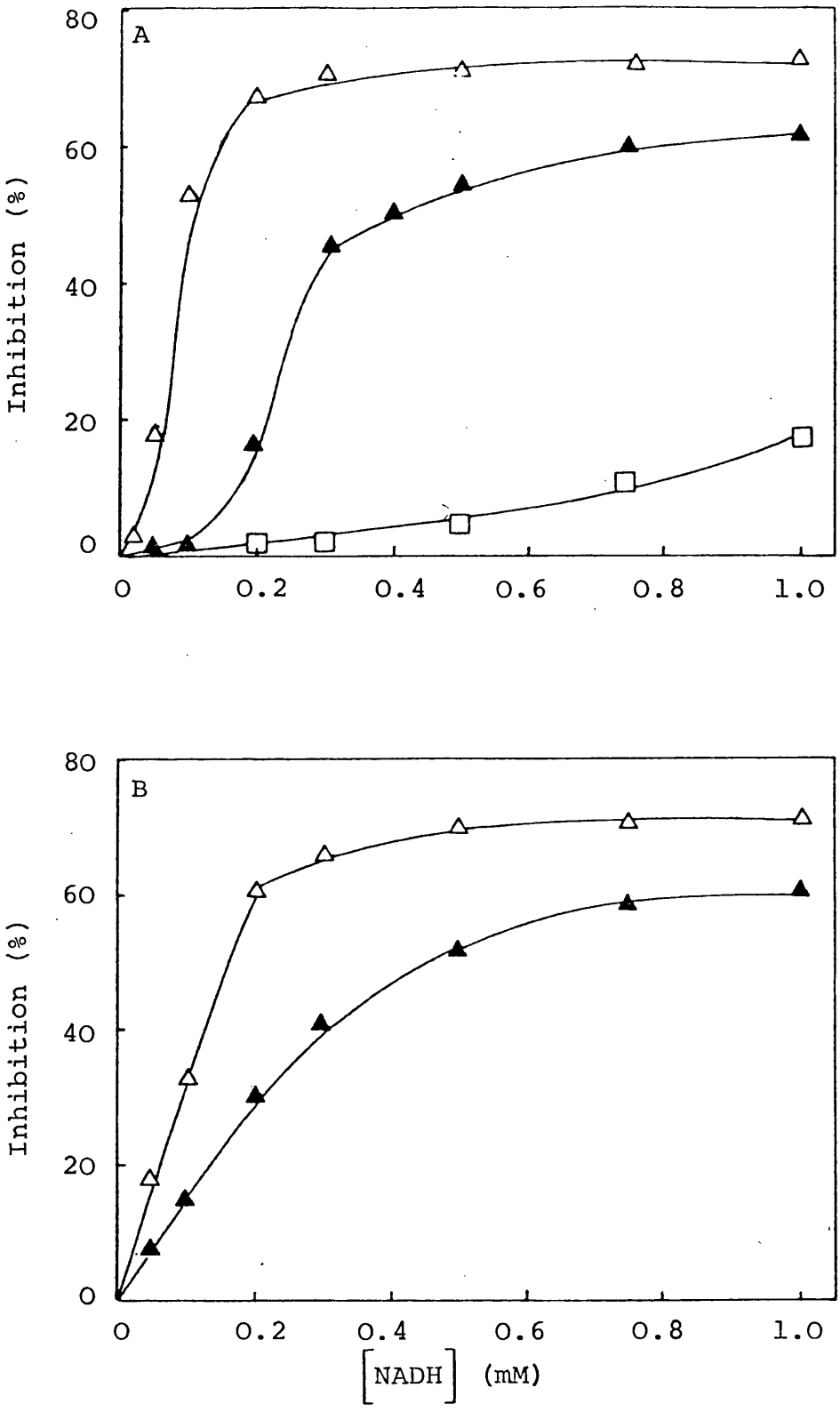


Fig.37. Inhibition of wild-type Ps.aeruginosa citrate synthase (A) and mutant CS I (B) by NADH
Enzyme activity was measured using assay Method 1.
(△) In 'Tris buffer (pH 8.0)';
(▲) In 'Tris buffer (pH 8.0)' containing 0.1 M-KCl.
(□) Enzyme desensitized by incubation with 0.1 mM-DTNB for 5 min in 'Tris buffer (pH 8.0)'.

was probably due to the oxidation of thiol groups on the enzyme and, under these conditions, only the non-specific isosteric nucleotide inhibition was observed. Oxidation of the thiol groups was minimised by purification and storage of the enzyme in 'Tris buffer (pH 7.0)'.

CS I was also inhibited by NADH. However, in this case, there was no sigmoidicity. Fig.38 shows the effect of AMP on NADH-inhibited wild-type citrate synthase and mutant CS I. In order to distinguish between activation of CS I by AMP and deinhibition of the NADH-inhibited enzyme it was necessary to determine the NADH inhibition at a fixed concentration of AMP (Fig.39). The inhibition of the enzyme was identical to that seen in the absence of AMP (Fig.37). This indicated that CS I was activated by AMP and inhibited by NADH, but that AMP did not affect the NADH inhibition of the enzyme. These findings suggested that AMP and NADH had separate binding sites on the enzyme.

Wild-type citrate synthase was inhibited by NADH but was not activated by AMP; the effect of AMP was seen only in the presence of NADH. Fig.39 shows that AMP overcame the inhibition by NADH but the results did not indicate whether AMP and NADH had the same, or different, binding sites on the enzyme.

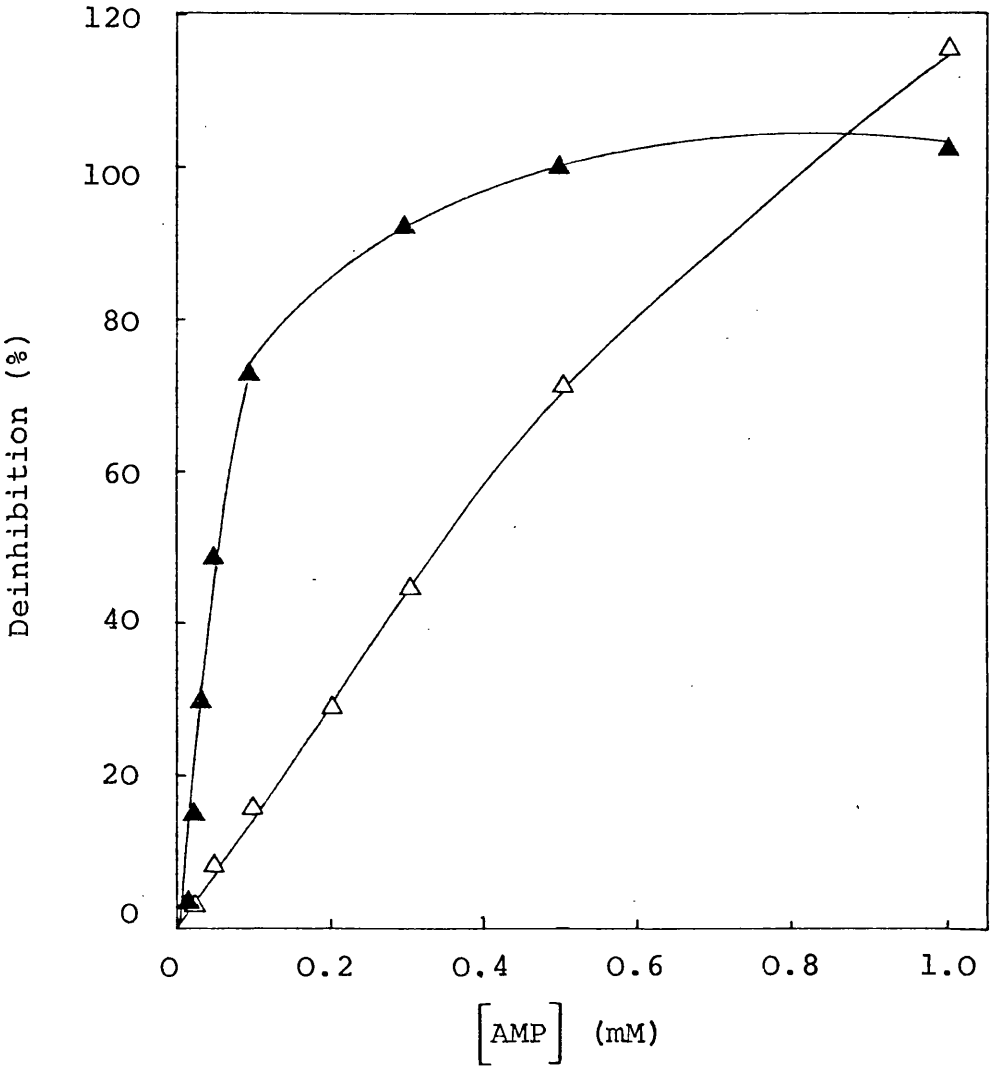


Fig.38. Deinhibition by AMP of NADH-inhibited wild-type Ps.aeruginosa citrate synthase and mutant CS I

Enzyme activity was measured using assay Method 1 in 'Tris buffer (pH 8.0)' in the presence of 0.2 mM-NADH.
(▲) Wild-type citrate synthase;
(△) Mutant CS I.

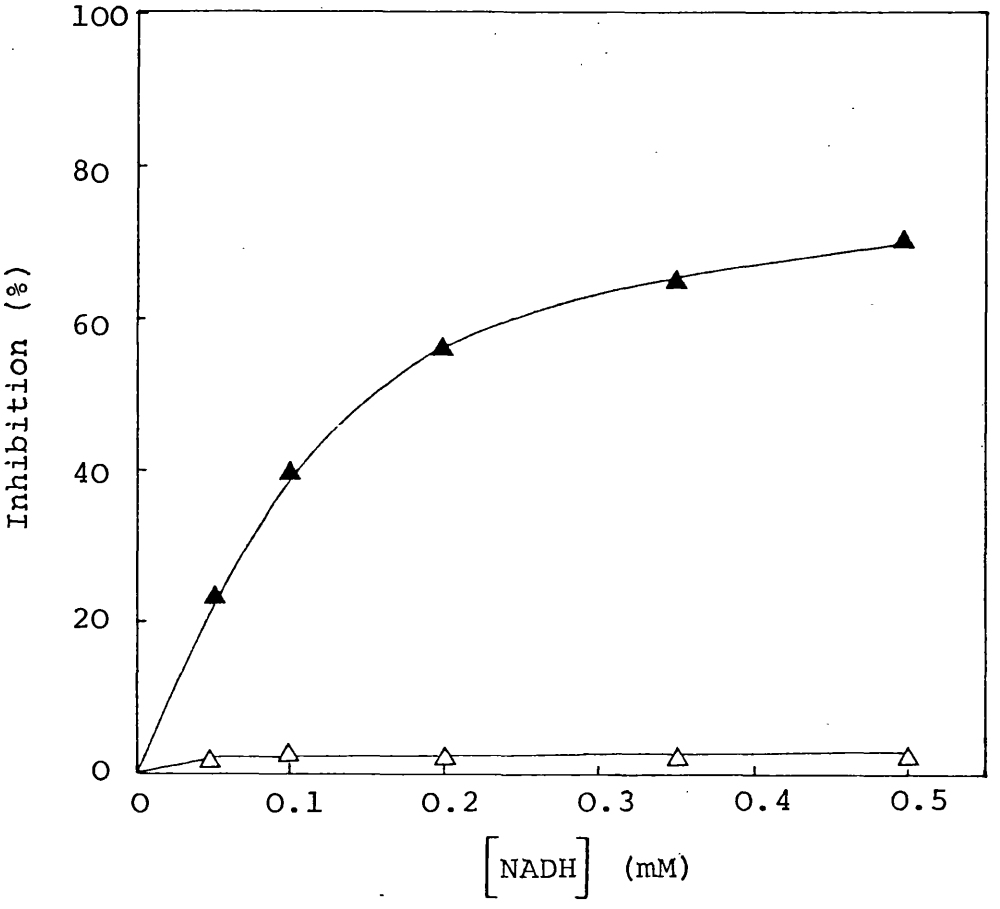


Fig.39. Effect of AMP on the NADH inhibition of wild-type Ps.aeruginosa citrate synthase and mutant CS I

Enzyme activity was measured using assay Method 1 in 'Tris buffer (pH 8.0)' in the presence of 0.5 mM-AMP.
(△) Wild-type citrate synthase;
(▲) Mutant CS I.

pH Dependence

During the purification of mutant CS I it was shown that the enzyme was more stable and more active at pH 7.0 than at pH 8.0. It also appeared to be more sensitive to AMP and NADH at the lower pH. This prompted an investigation of the pH dependences of both activity and sensitivity to effectors for the three enzymes used in this study.

Fig.40 shows the pH dependences of the three enzyme activities. The assays were performed polarographically because the DTNB method could only be used over a limited pH range. The wild-type citrate synthase had a pH optimum of 7.5-8.0, whereas CS I had an optimum at pH 7.0. CS II had an optimum pH 7.5-8.5 which was fairly close to that of the wild-type enzyme.

The wild-type enzyme showed a very broad pH dependence of NADH inhibition; the optimum at pH 7.0 was identical to that of CS I (Fig.41). CS II was not affected by NADH. AMP activation of CS I was also maximal at about pH 7.0.

Thermal Inactivation

The thermal inactivation of wild-type and mutant

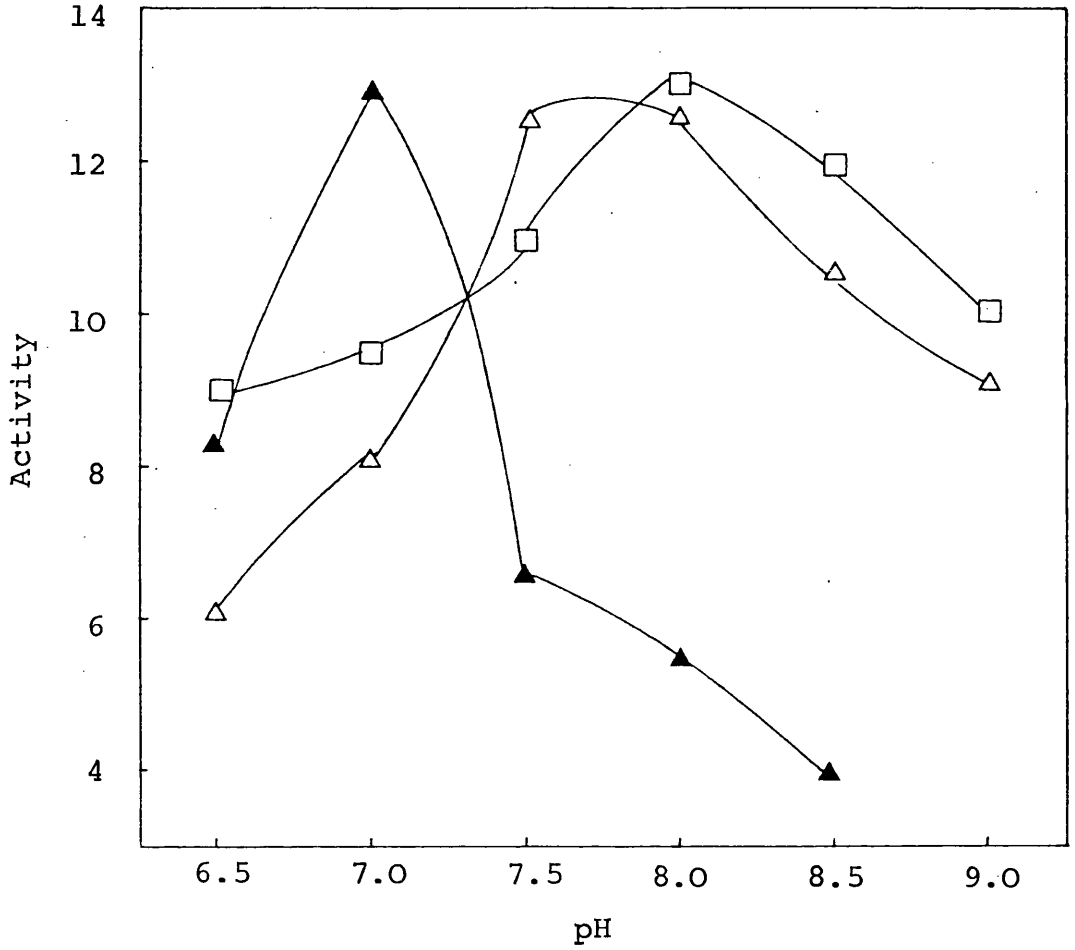


Fig.40. pH Dependence of wild-type and mutant Ps.aeruginosa citrate synthase activity

Enzyme activity (in arbitrary units) was measured using assay Method 2 in the presence of 0.16 mM-oxaloacetate and 0.14 mM-acetyl-CoA in buffers of composition 20 mM-Tris-HCl, 1 mM-EDTA.

(△) Wild-type citrate synthase;
(▲) Mutant CS I;
(□) Mutant CS II.

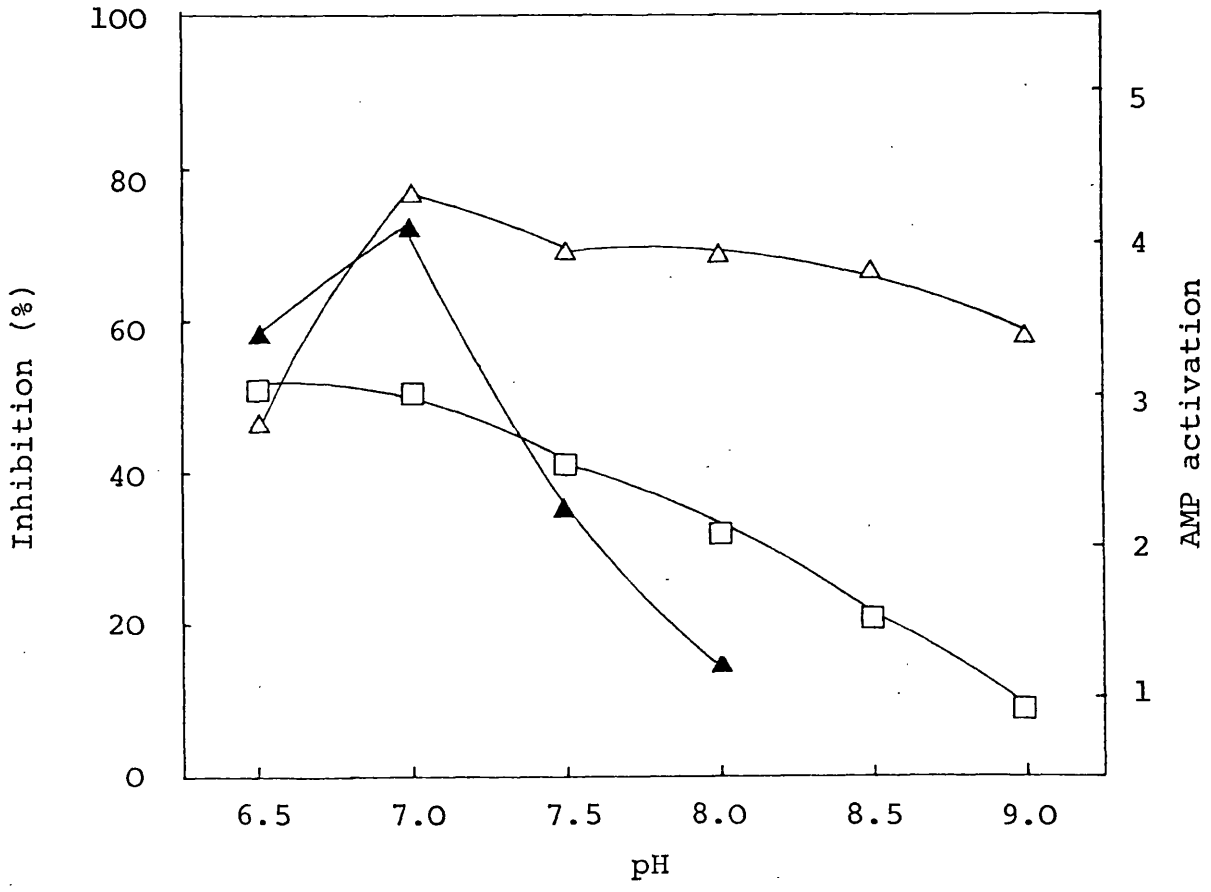


Fig.41. pH Dependence of the NADH inhibition of wild-type Ps.aeruginosa citrate synthase and mutant CS I and of the AMP activation of mutant CS I

Enzyme activity was measured using assay Method 2 in the presence of 0.16 mM-oxaloacetate and 0.14 mM-acetyl-CoA in buffers of composition 20 mM-Tris-HCl, 1 mM-EDTA. NADH inhibition measured in the presence of 0.5 mM-NADH.

(△) Wild-type citrate synthase;
(▲) Mutant CS I;
(□) AMP activation of mutant CS I at a concentration of 0.5 mM-AMP (in the absence of NADH).

Ps.aeruginosa citrate synthases was determined in the presence and absence of substrates and a number of effectors. Fig.42 compares the thermal inactivation of the three enzymes. The wild-type enzyme was slightly more stable than the two mutant enzymes.

The inactivation of the wild-type enzyme was reduced in the presence of substrates and the effectors AMP and NADH (Fig.43). The specificity of these effectors was shown by the fact that NADPH, $NADP^+$, NAD^+, ADP and ATP conferred no protection on the enzyme (Fig.44). The rate of thermal inactivation was also reduced in the presence of 0.1 M-KCl.

If the wild-type enzyme was stored in 'Tris buffer (pH 8.0)' it lost its NADH sensitivity. Fig.45 shows that the enzyme was no longer protected from thermal inactivation by NADH but there was still considerable protection by oxaloacetate. Treatment of the enzyme with 0.1 mM-DTNB for 5 min resulted in loss of NADH inhibition. The rate of thermal inactivation was enhanced in the presence of DTNB. There was some protection by oxaloacetate but none by NADH. Excess DTNB and free TNB^- were removed by filtering the enzyme through a small column of Sephadex G-25. The thermal inactivation

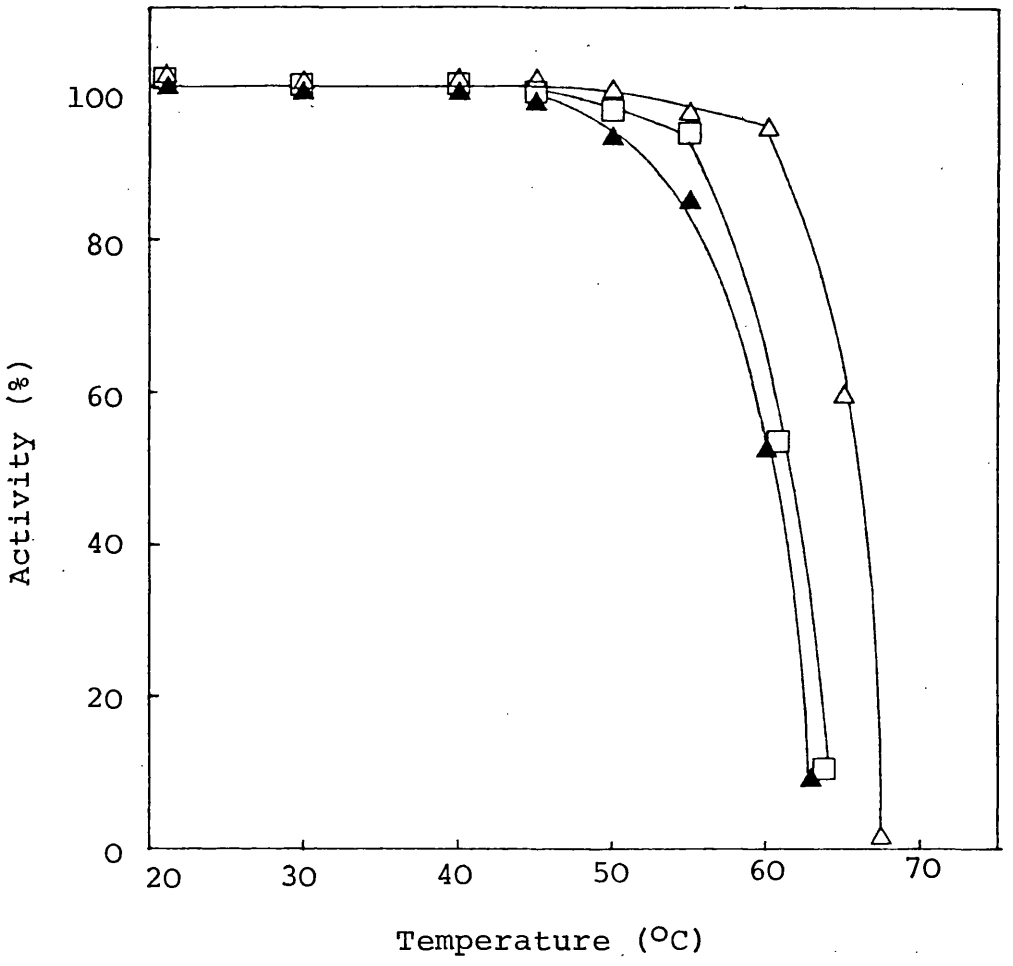


Fig.42. Thermal inactivation of wild-type and mutant Ps.aeruginosa citrate synthases

Enzyme activity was measured using assay Method 1 after incubation for 5 min at various temperatures.
(△) Wild-type citrate synthase;
(▲) Mutant CS I;
(□) Mutant CS II..

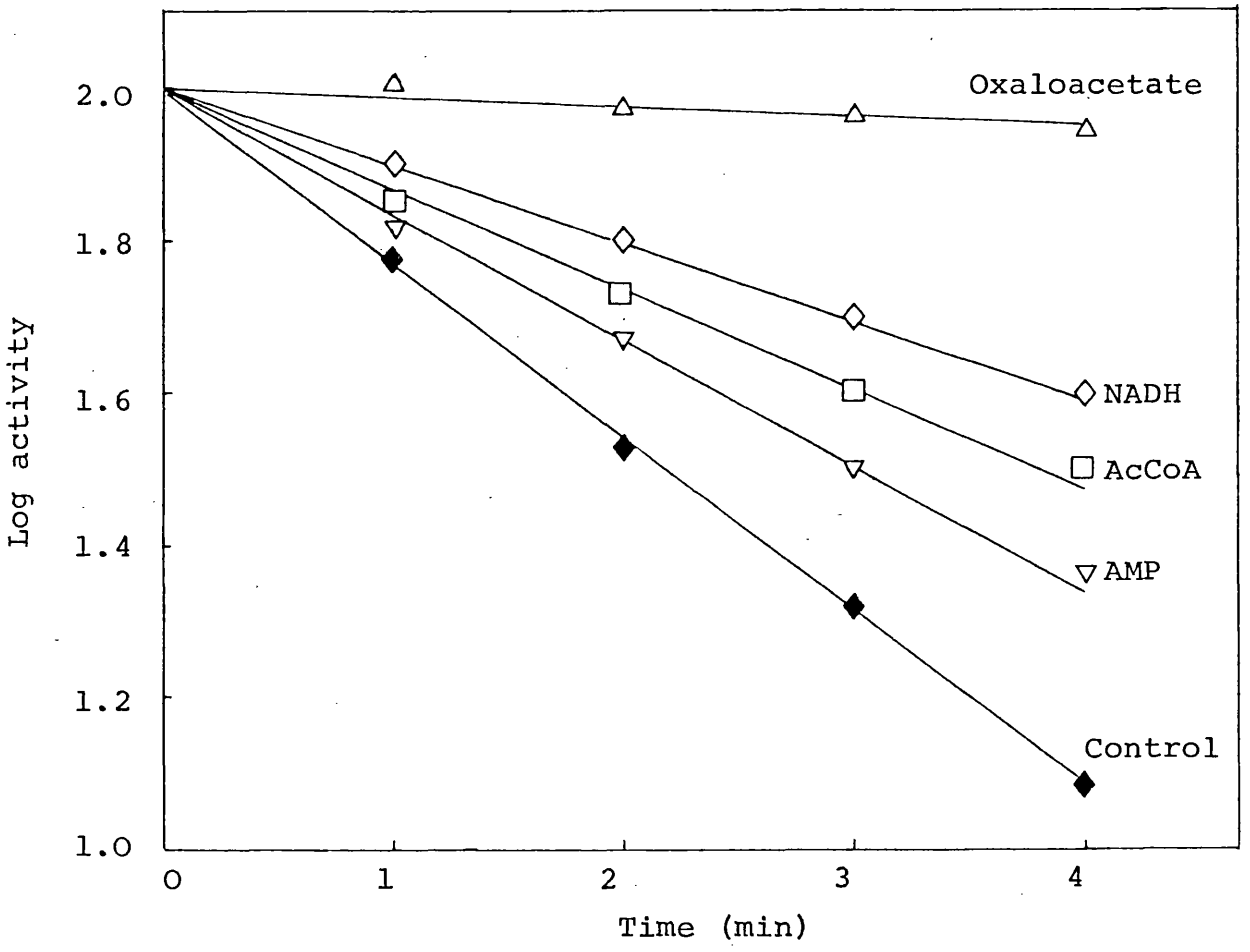


Fig.43. Thermal inactivation of wild-type Ps.aeruginosa citrate synthase in the presence and absence of substrates and effectors.
All metabolites were used at a concentration of 1 mM. The temperature of incubation was 65°C. Enzyme activity was measured using assay Method 1.

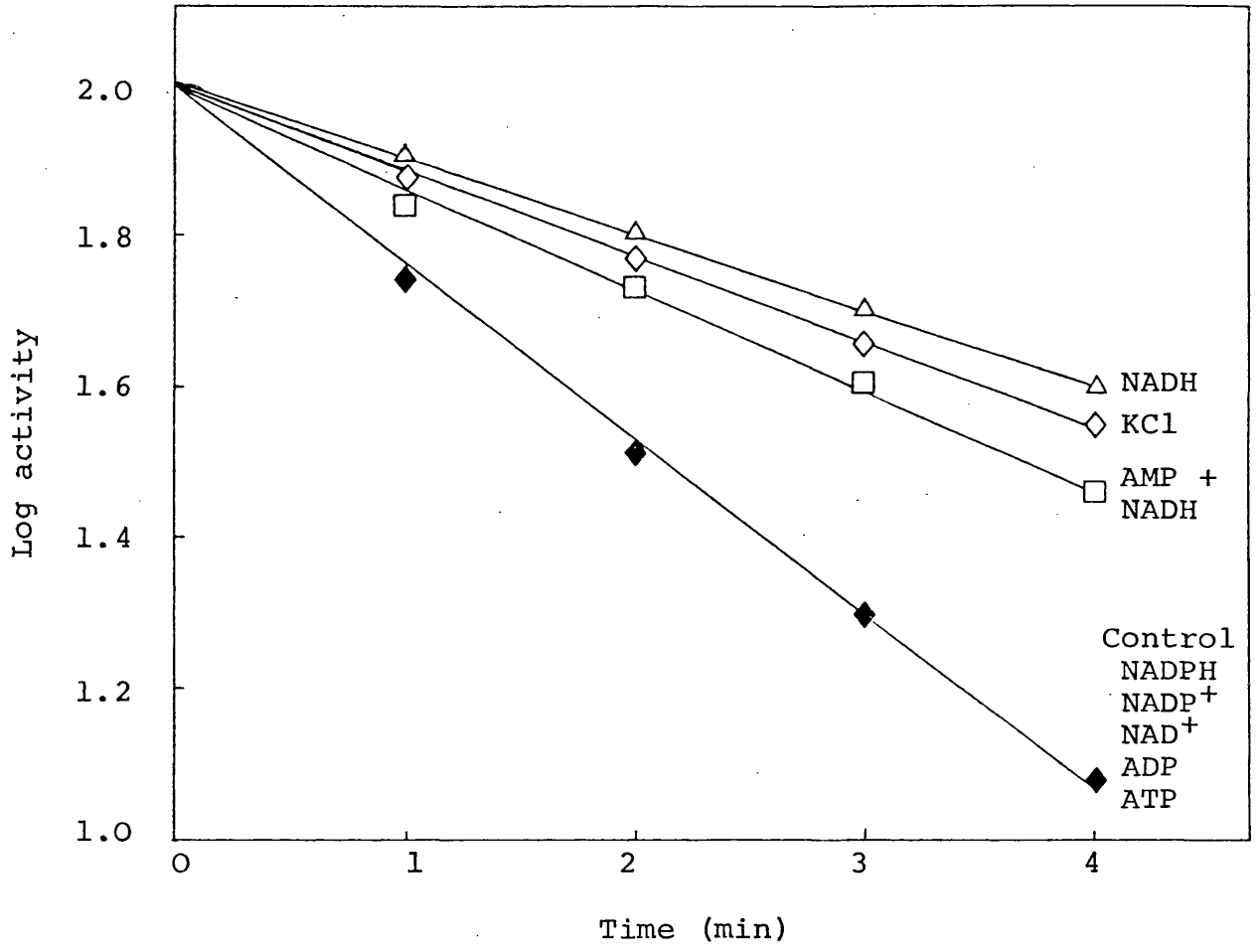


Fig.44. Thermal inactivation of wild-type Ps.aeruginosa citrate synthase in the presence and absence of effectors
KCl was used at a concentration of 0.1 M.
All metabolites were used at a concentration of 1 mM.
The temperature of incubation was 65°C.
Enzyme activity was measured using assay Method 1.

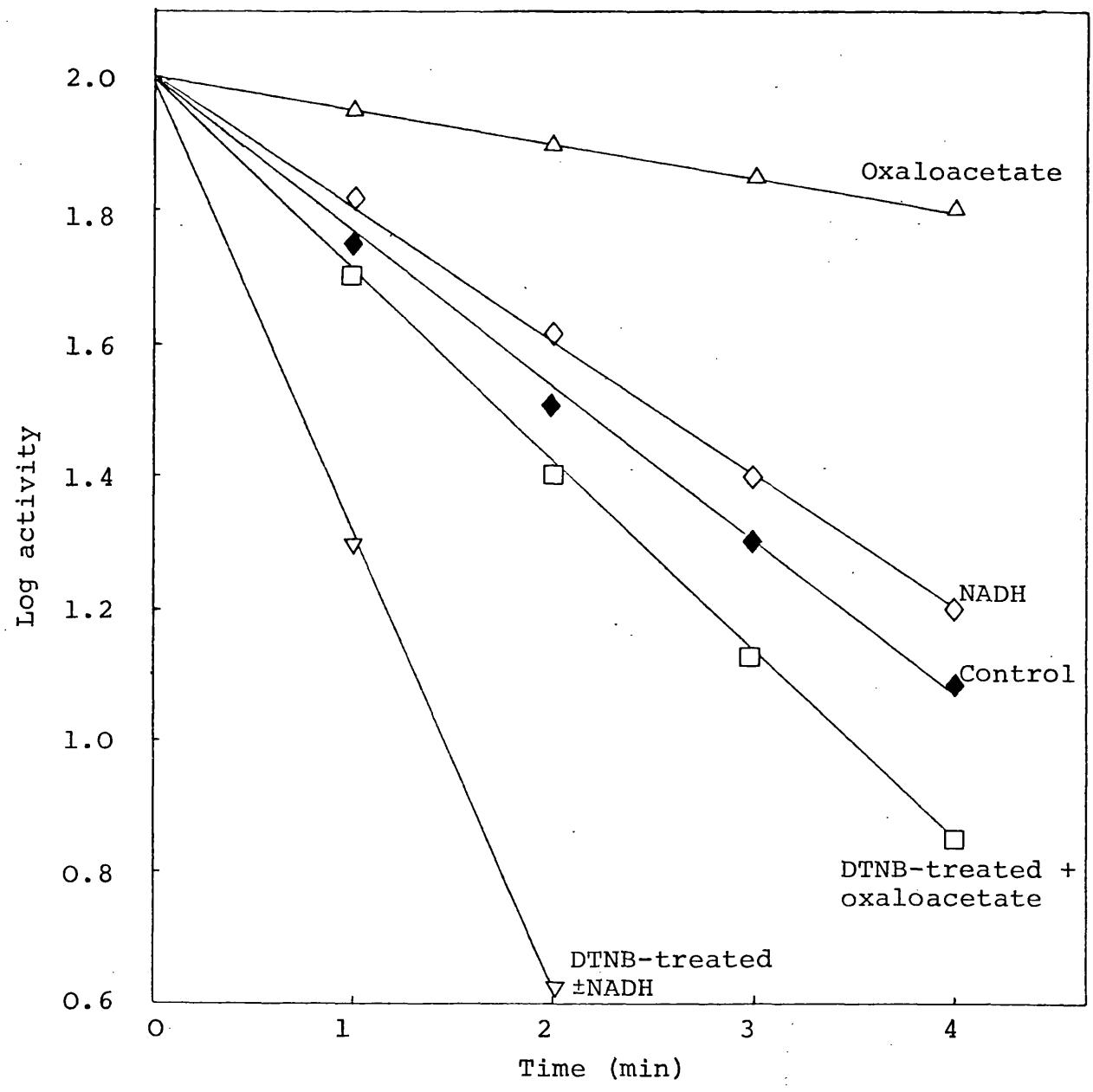


Fig.45. Thermal inactivation of DTNB-treated and NADH-insensitive wild-type Ps.aeruginosa citrate synthase in the presence and absence of substrates and effectors
All metabolites were used at a concentration of 1 mM.
The temperature of incubation was 65°C.
Enzyme activity was measured using assay Method 1.

studies were repeated and the results obtained were identical to those in the presence of excess unreacted DTNB. Therefore the binding of TNB^- to certain thiol groups altered the thermal stability of the wild-type enzyme.

The mutant CS I was also protected against thermal inactivation by its substrates and the effectors AMP, ADP and NADH. The protection by AMP was greater than that observed with the wild-type enzyme. Treatment of the enzyme with DTNB did not affect the thermal stability or the protection by NADH (Fig.46).

Mutant CS II was protected against thermal inactivation by acetyl-CoA, oxaloacetate and 0.1 M-KCl but not by AMP or NADH (Fig.47).

Photo-Oxidation

Photo-oxidation has been used previously in the investigation of functional groups involved in the catalytic and regulatory properties of citrate synthase from E.coli (Danson and Weitzman, 1973) and A.calcoaceticus (Weitzman et al., 1974). In order to determine the groups involved in the catalytic and regulatory properties of citrate synthase it is necessary to have enzyme of high

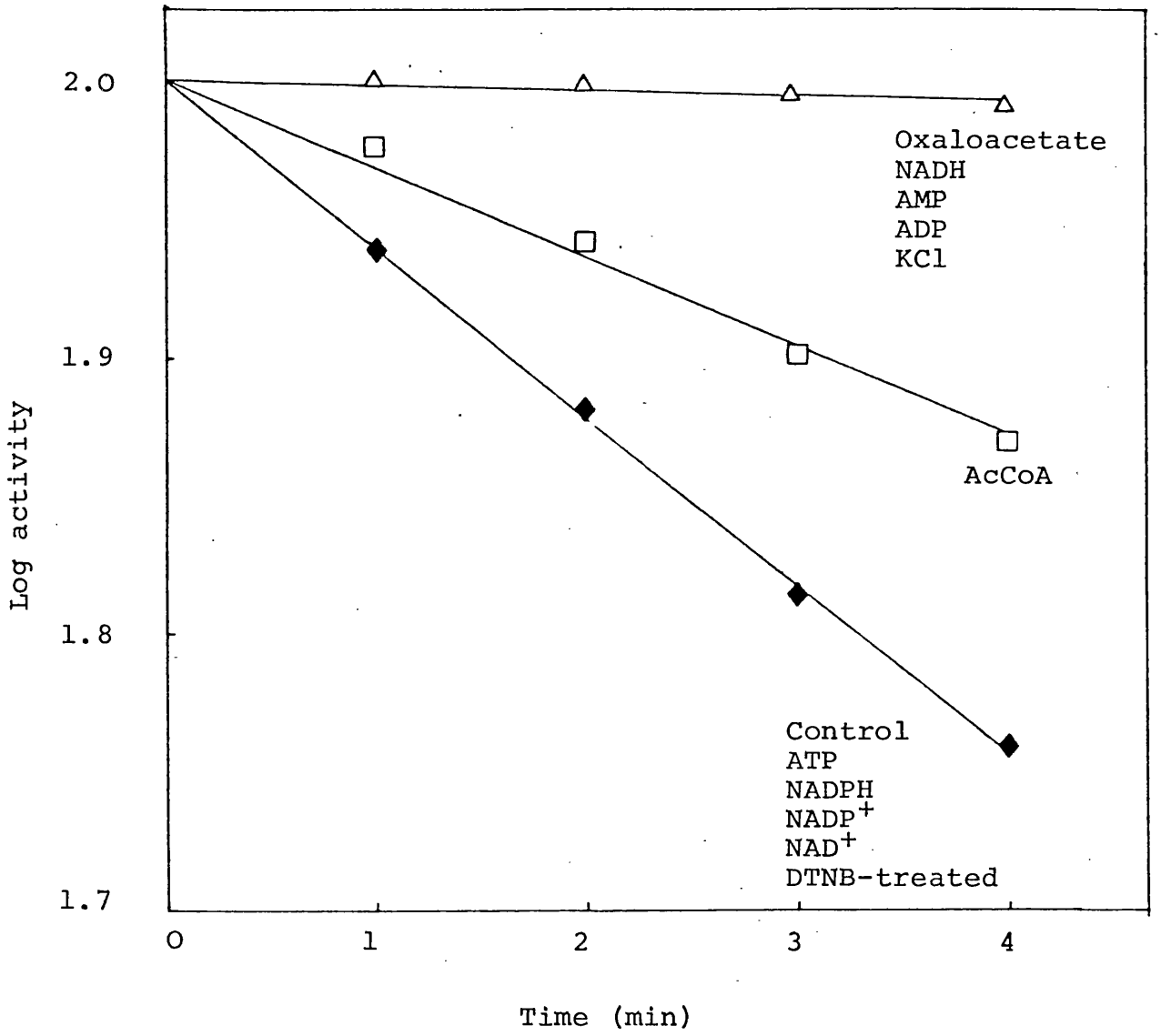


Fig.46. Thermal inactivation of mutant Ps.aeruginosa CS I in the presence and absence of substrates and effectors
KCl was used at a concentration of 0.1 M.
All metabolites were used at a concentration of 1 mM.
The temperature of incubation was 60°C.
Enzyme activity was measured using assay Method 1.

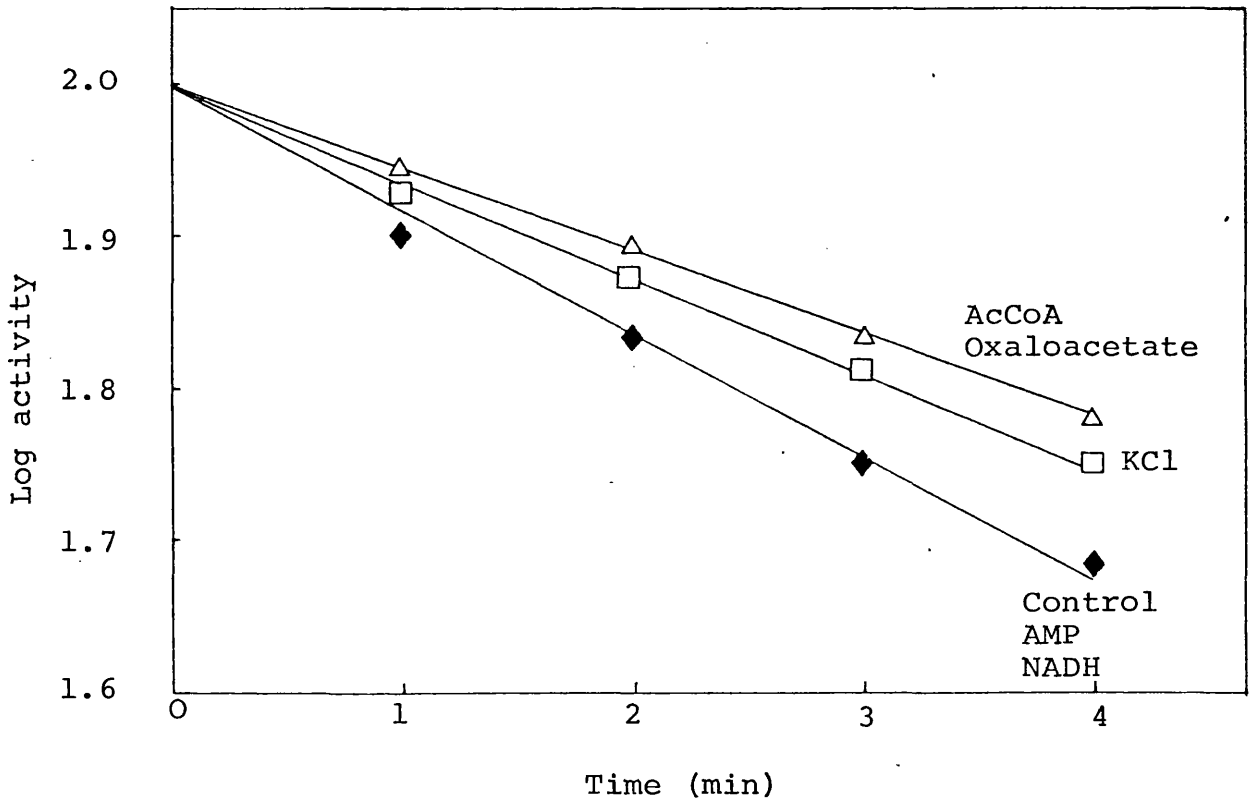


Fig.47. Thermal inactivation of mutant Ps.aeruginosa CS II in the presence and absence of substrates and effectors
KCl was used at a concentration of 0.1 M.
All metabolites were used at a concentration of 1 mM.
The temperature of incubation was 60°C.
Enzyme activity was measured using assay Method 1.

purity and in sufficient quantity to enable a number of determinations at different pH's to be made. This was not the case in this study, so the technique was used to probe differential rates of loss of activity and loss of NADH and AMP regulation.

Fig.48 shows the effect of photo-oxidation on wild-type Ps.aeruginosa citrate synthase in the presence of the photo-sensitive dye Rose Bengal. The presence of 1 mM-oxaloacetate resulted in considerable protection of the enzyme against photo-oxidative destruction of activity and this enabled the study of selective photo-oxidation of the regulatory sensitivity to NADH. Photo-oxidation resulted in complete desensitization to NADH inhibition (Fig.49).

Mutant Ps.aeruginosa CS I was also inactivated by photo-oxidation in the presence of the anionic dye Rose Bengal and the cationic dye Methylene Blue (Fig.50). This time there was very little protection against loss of activity by oxaloacetate. This was probably due to the fact that the enzyme had a very high $S_{0.5}$ for oxaloacetate compared with the wild-type enzyme and the substrate was therefore not present at saturating levels.

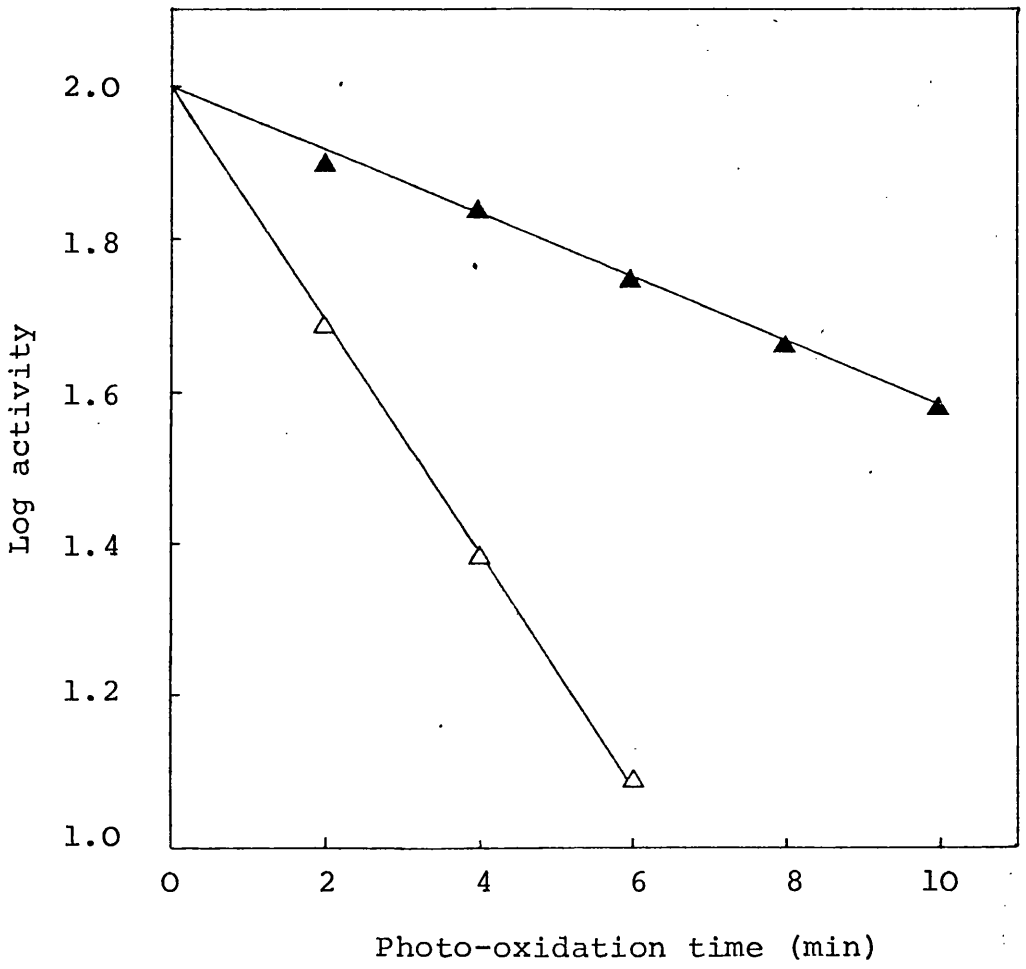


Fig.48. Photo-inactivation of wild-type Ps.aeruginosa citrate synthase with Rose Bengal (3 μM).
Photo-oxidation was carried out in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme alone;
(▲) Enzyme in the presence of 1 mM-oxaloacetate.

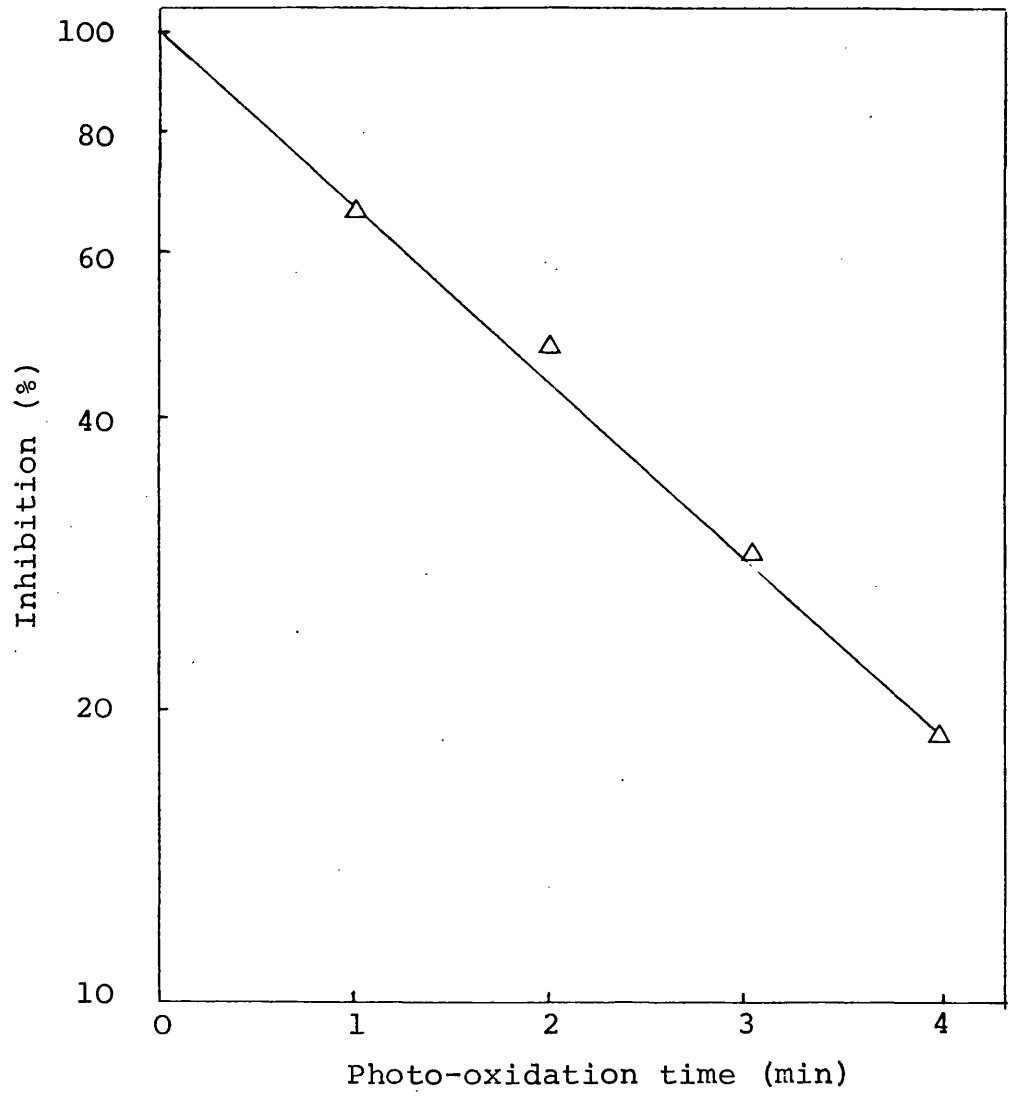


Fig.49. Effect of photo-oxidation on the inhibition of wild-type Ps.aeruginosa citrate synthase activity by NADH
Photo-oxidation was carried out in 'Tris buffer (pH 8.0)' in the presence of 3 μM-Rose Bengal. NADH inhibition (expressed as % of initial value) was measured using assay Method 1.

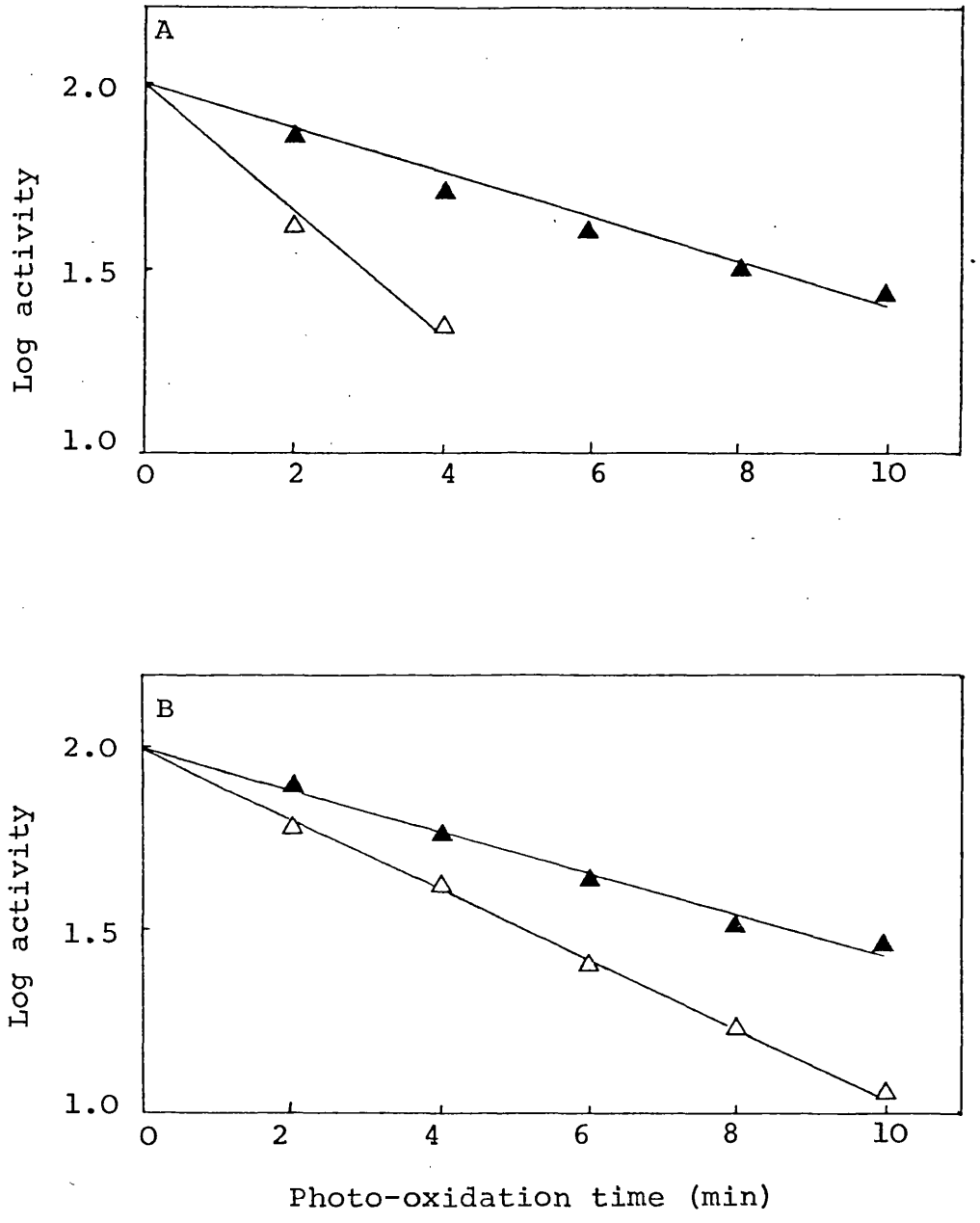


Fig.50. Comparison of the photo-inactivation of mutant Ps.aeruginosa CS I with Methylene Blue and Rose Bengal

Photo-oxidation was carried out in 'Tris buffer (pH 8.0)' in the presence of 1.3 μM-Methylene Blue (curve A) and 1.3 μM-Rose Bengal (curve B). Enzyme activity was measured using assay Method 1.
(△) Enzyme alone;
(▲) Enzyme in the presence of 1 mM-oxaloacetate.

Photo-oxidation of mutant CS I resulted in a rapid loss of sensitivity to NADH in the presence of Methylene Blue with a much slower loss of AMP activation (Fig.51). This desensitization to NADH was much more marked than with Rose Bengal and presented further evidence for different binding sites for AMP and NADH.

The effect of photo-oxidation on the activity of mutant CS II was also investigated (Fig.52). Oxaloacetate conferred considerable protection against loss of activity as is also the case with the wild-type enzyme.

Treatment with Specific Thiol-Blocking Reagents

Particular attention has been paid to the specific covalent modification of thiol groups in citrate synthase. The effects of thiol modification on the activity and regulation of bacterial citrate synthases depend on the organisms of origin (Weitzman and Danson, 1976).

It has been seen that wild-type Ps.aeruginosa citrate synthase readily lost its sensitivity to NADH inhibition when stored in 'Tris buffer (pH 8.0)' and it was thought that this may be due to the oxidation of thiol groups.

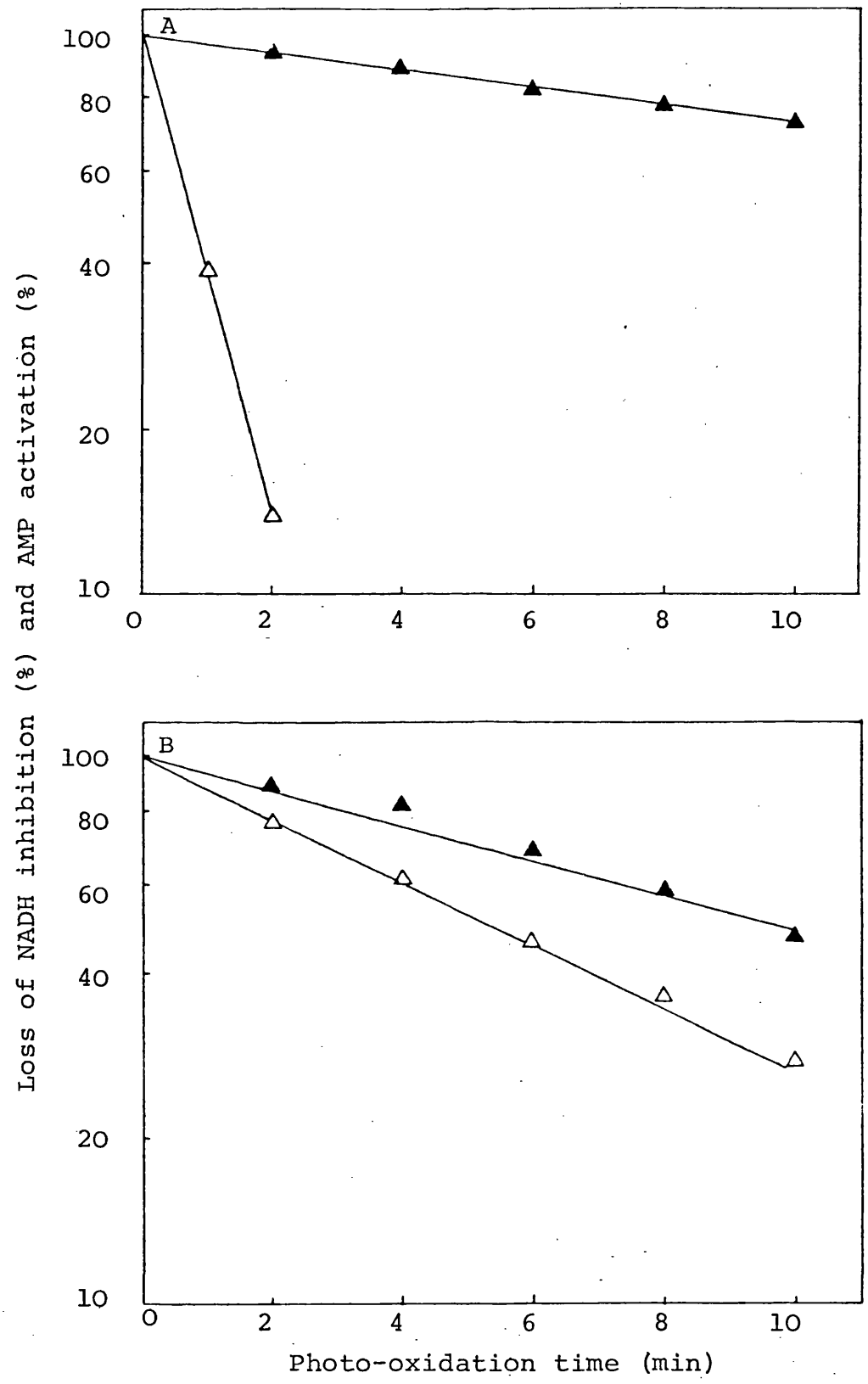


Fig.51. Comparison of the effects of photo-oxidation on the NADH inhibition and AMP activation of mutant Ps.aeruginosa CS I by Methylene Blue and Rose Bengal

Photo-oxidation was carried out in 'Tris buffer (pH 8.0)' in the presence of 1.3 μM-Methylene Blue (curve A) and 1.3 μM-Rose Bengal (curve B).
Enzyme activity was measured using assay Method 1.
(△) NADH inhibition (expressed as % of initial value);
(▲) AMP activation (expressed as % of initial value).

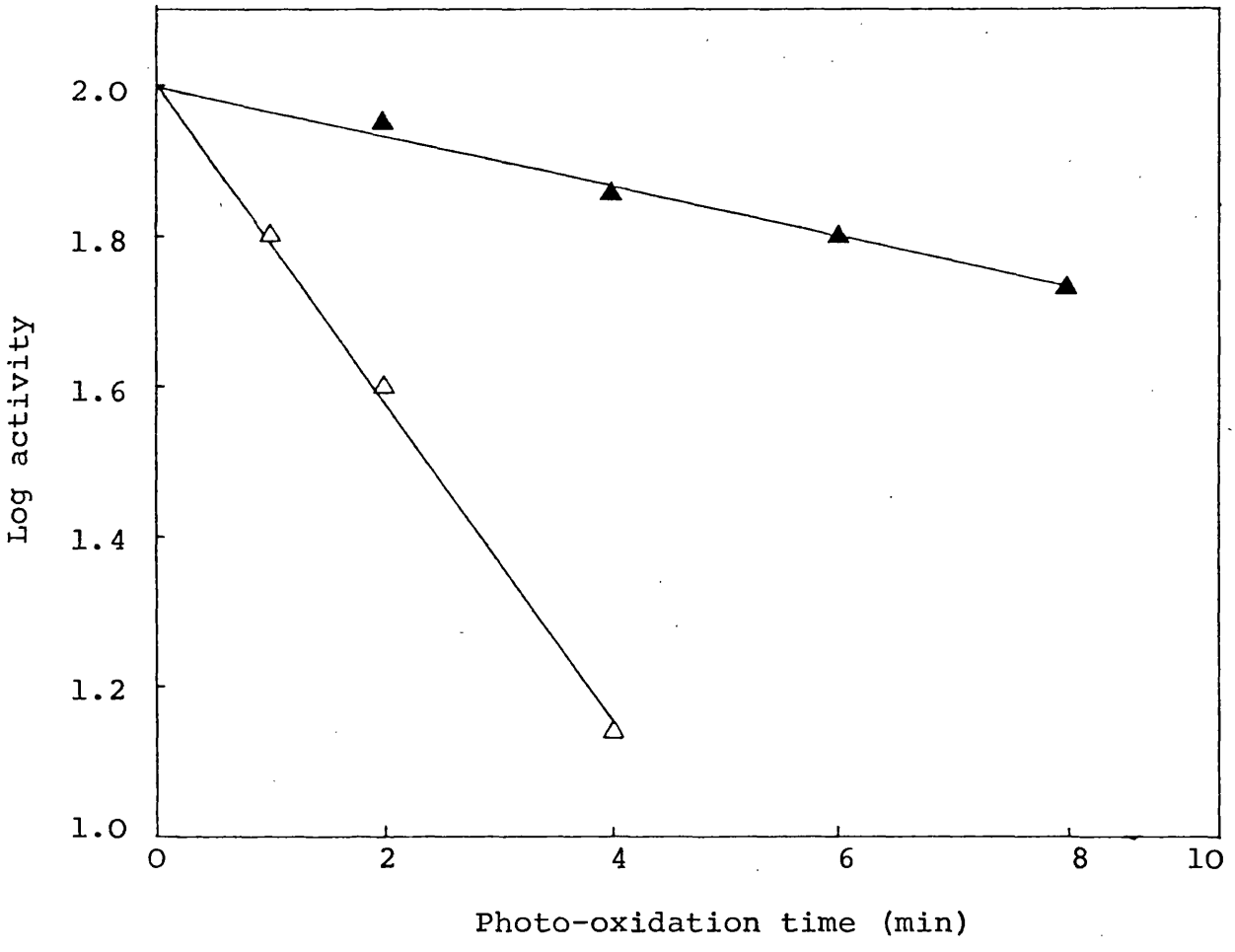


Fig.52. Photo-oxidation of mutant Ps.aeruginosa CS II with Rose Bengal (3 µM)
Photo-oxidation was carried out in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme alone;
(▲) Enzyme in the presence of 1 mM-oxaloacetate.

The enzyme was treated with the thiol-blocking reagent DTNB (0.1 mM) and the effects on activity (Fig.53) and NADH inhibition (Fig.54) were investigated.

The wild-type enzyme was slowly inactivated by DTNB with almost complete protection by the substrate oxaloacetate (Fig. 53), whereas the enzyme was rapidly desensitized to inhibition by NADH with no protection by oxaloacetate (Fig.54). Some protection was conferred by the effectors AMP and NADH. This indicated that there were at least two distinct sets of thiol groups one of which was involved in the activity of the enzyme and the other in the response to NADH. Sensitivity to NADH was restored by treatment of the modified enzyme with 0.1 mM-dithiothreitol.

The mutant Ps.aeruginosa CS I was more rapidly inactivated by DTNB than was the wild-type enzyme (Fig.55). There was no protection by oxaloacetate or AMP. Fig.56 shows the effect of DTNB on the NADH inhibition and AMP activation of CS I. There was a slight decrease in the activation by AMP and complete desensitization to inhibition by NADH. DTNB had no effect on the activity of CS II even when used at a concentration of 0.4 mM.

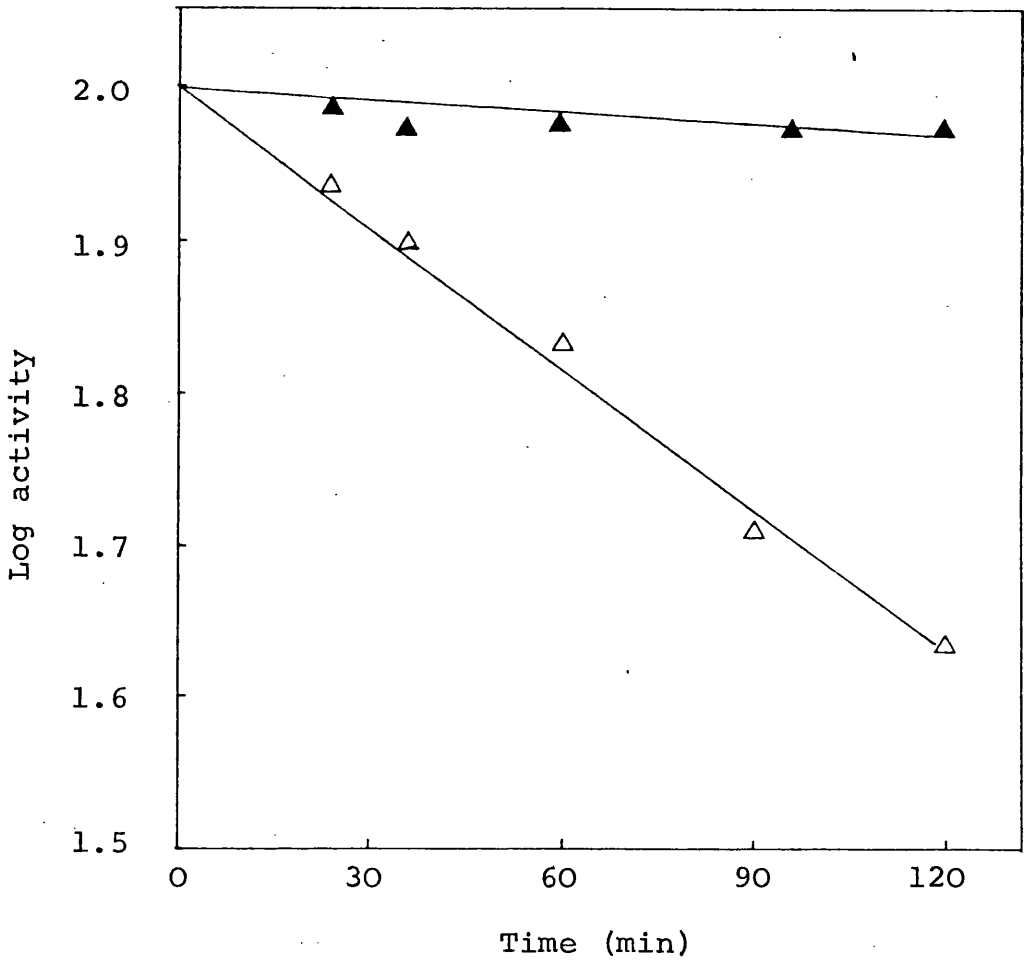


Fig.53. Effect of DTNB on the activity of wild-type Ps.aeruginosa citrate synthase

Enzyme was incubated at 20°C with 0.1 mM-DTNB in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme alone;
(▲) Enzyme in the presence of 0.2 mM-oxaloacetate.

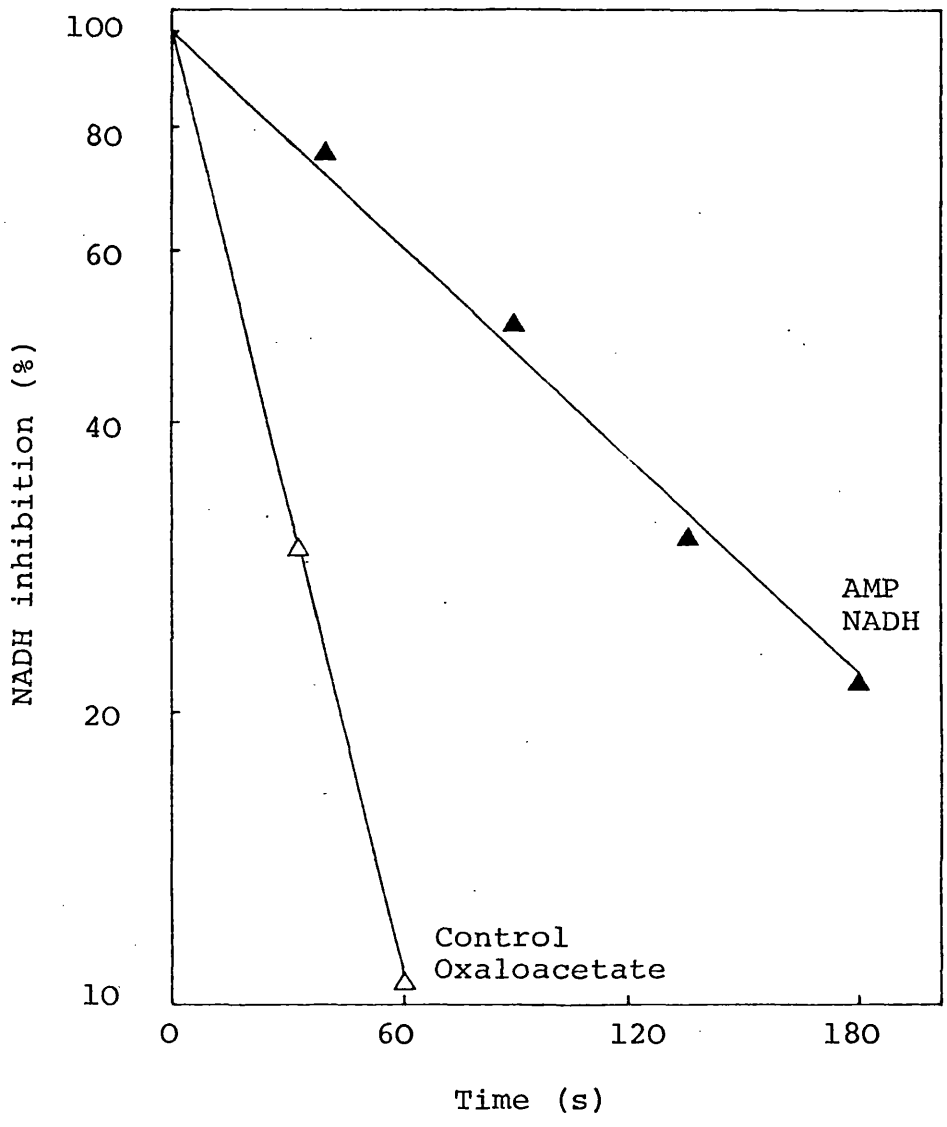


Fig.54. Effect of DTNB on the NADH inhibition of wild-type Ps.aeruginosa citrate synthase in the presence and absence of substrate and effectors
Enzyme was incubated at 20°C with 0.1 mM-DTNB in 'Tris buffer (pH 8.0)'.
All metabolites were used at a concentration of 0.1 mM.
NADH inhibition (expressed as % of initial value) was measured using assay Method 1.

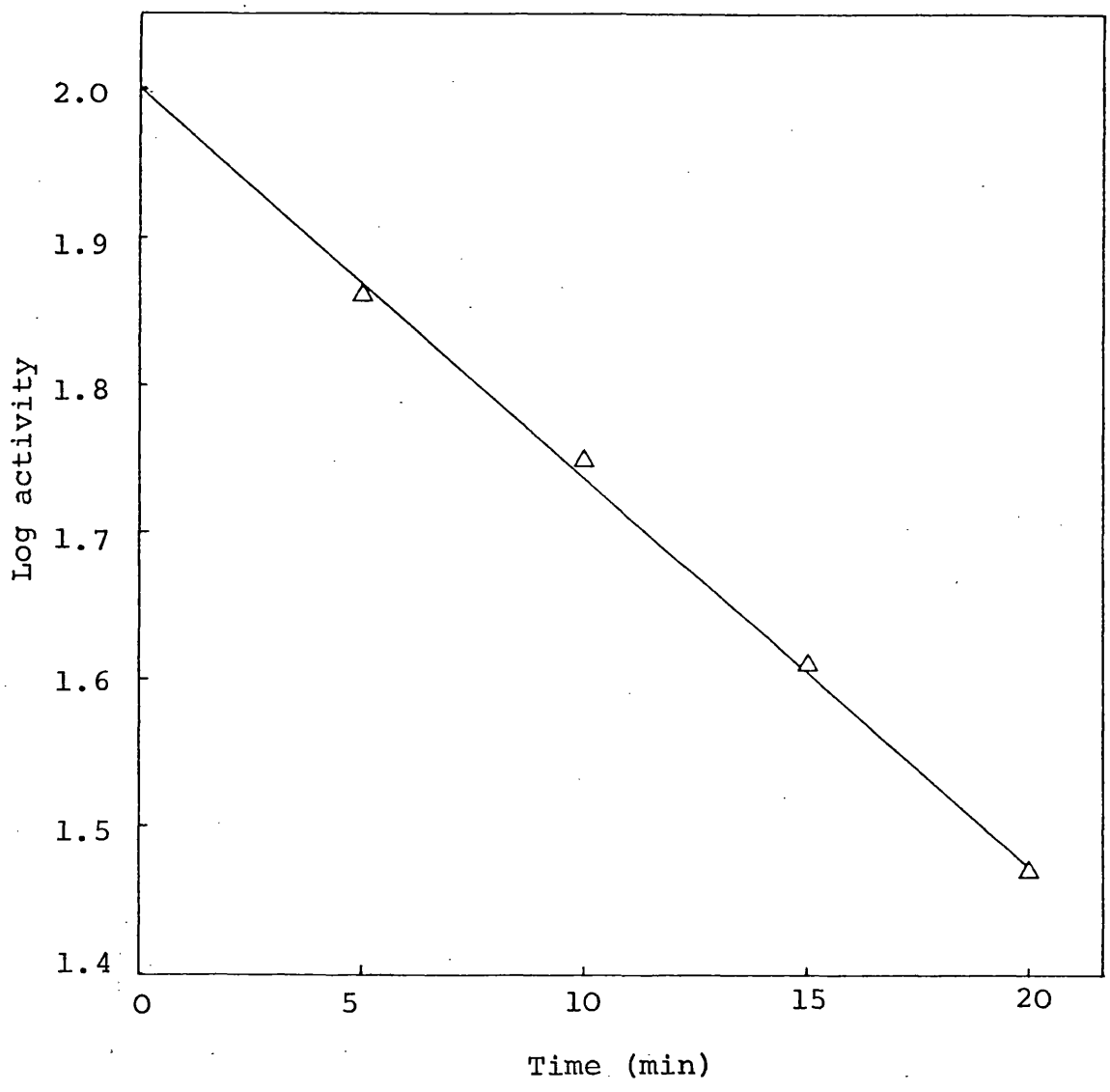


Fig.55. Effect of DTNB on the activity of mutant Ps.aeruginosa CS I
Enzyme was incubated at 20°C with 0.1 mM-DTNB in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.

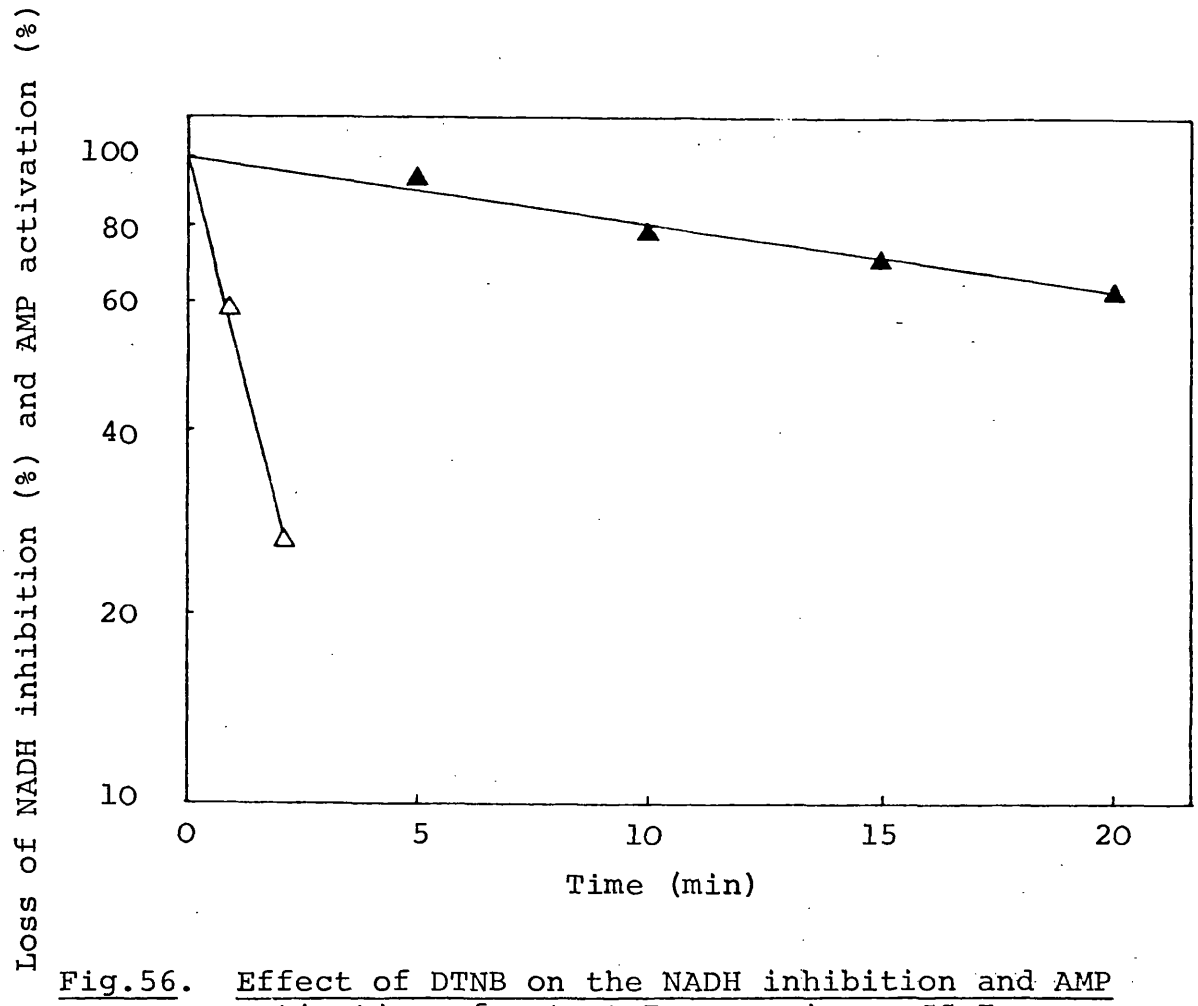


Fig.56. Effect of DTNB on the NADH inhibition and AMP activation of mutant Ps.aeruginosa CS I

Enzyme was incubated at 20°C with 0.1 mM-DTNB in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) NADH inhibition (expressed as % of initial value);
(▲) AMP activation (expressed as % of initial value).

The effects of salt on the modification of thiol groups by DTNB in wild-type and mutant Ps.aeruginosa citrate synthases were investigated and compared with the effects produced by citrate synthases from two other bacteria - A.calcoaceticus and E.coli. Crude extracts of these latter two enzymes were used.

Inactivation by DTNB of wild-type Ps.aeruginosa citrate synthase was reduced in the presence of either 0.1 M-KCl or 10 mM-$MgCl_2$ (Fig.57). Both 0.1 M-KCl and 10 mM-$MgCl_2$ enhanced the rate of inactivation by DTNB of mutant Ps.aeruginosa CS I (Fig.58) and E.coli citrate synthase (Fig.59). Assaying the modified E.coli citrate synthase in the presence of 0.1 M-KCl revealed no apparent loss of enzyme activity. Neither mutant Ps.aeruginosa CS II nor A.calcoaceticus citrate synthases were inactivated by DTNB in the presence or absence of salt.

DTNB is one of a number of chromogenic disulphide reagents which react by thiol-disulphide interchange (Brocklehurst, 1979). It was decided to compare the effectiveness of some of these reagents on diverse citrate synthases. 4-PDS, 2-PDS, DTNP and DTNB were therefore used to modify wild-type and mutant Ps.aeruginosa citrate synthases and a comparison made with the behaviour of the E.coli, A.calcoaceticus and pig heart enzymes.

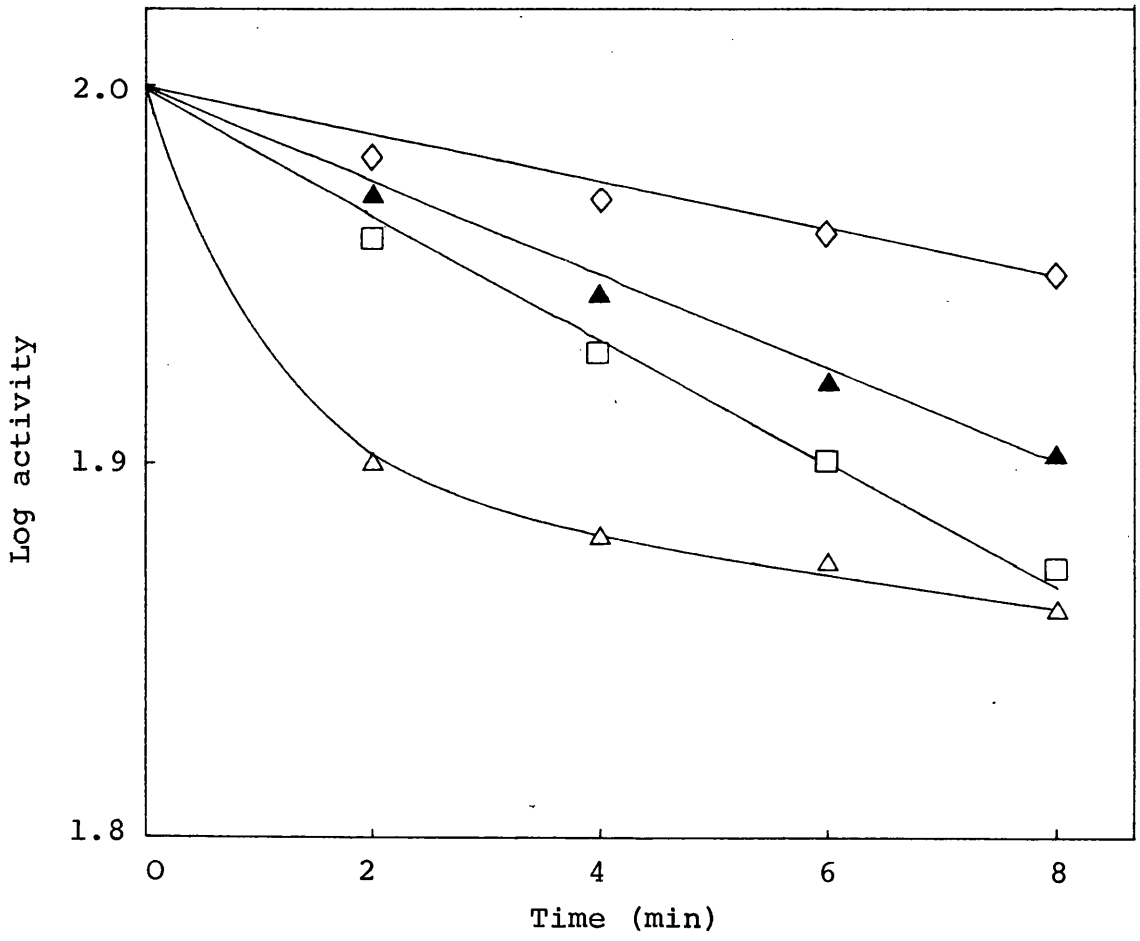


Fig.57. Effect of salt on the modification of wild-type Ps.aeruginosa citrate synthase by DTNB

Enzyme was incubated at 20°C with 0.1 mM-DTNB in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme assayed in the absence of 0.1 M-KCl;
(▲) Enzyme assayed in the presence of 0.1 M-KCl;
(◇) Enzyme modified in the presence of 0.1 M-KCl;
(□) Enzyme modified in the presence of 10 mM-$MgCl_2$.

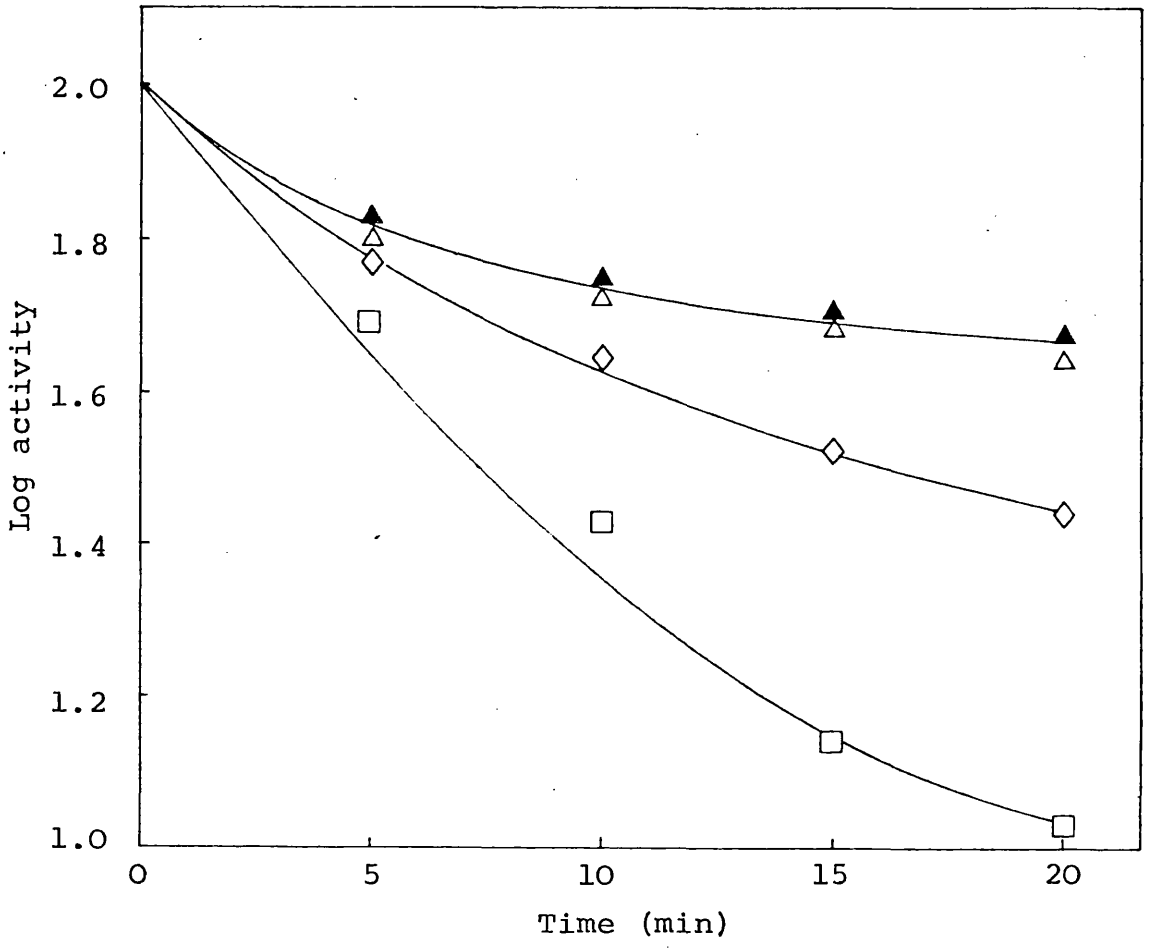


Fig.58. Effect of salt on the modification of mutant Ps.aeruginosa CS I by DTNB

Enzyme was incubated at 20°C with 0.1 mM-DTNB in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme assayed in the absence of 0.1 M-KCl;
(▲) Enzyme assayed in the presence of 0.1 M-KCl;
(◇) Enzyme modified in the presence of 0.1 M-KCl;
(□) Enzyme modified in the presence of 10 mM-$MgCl_2$.

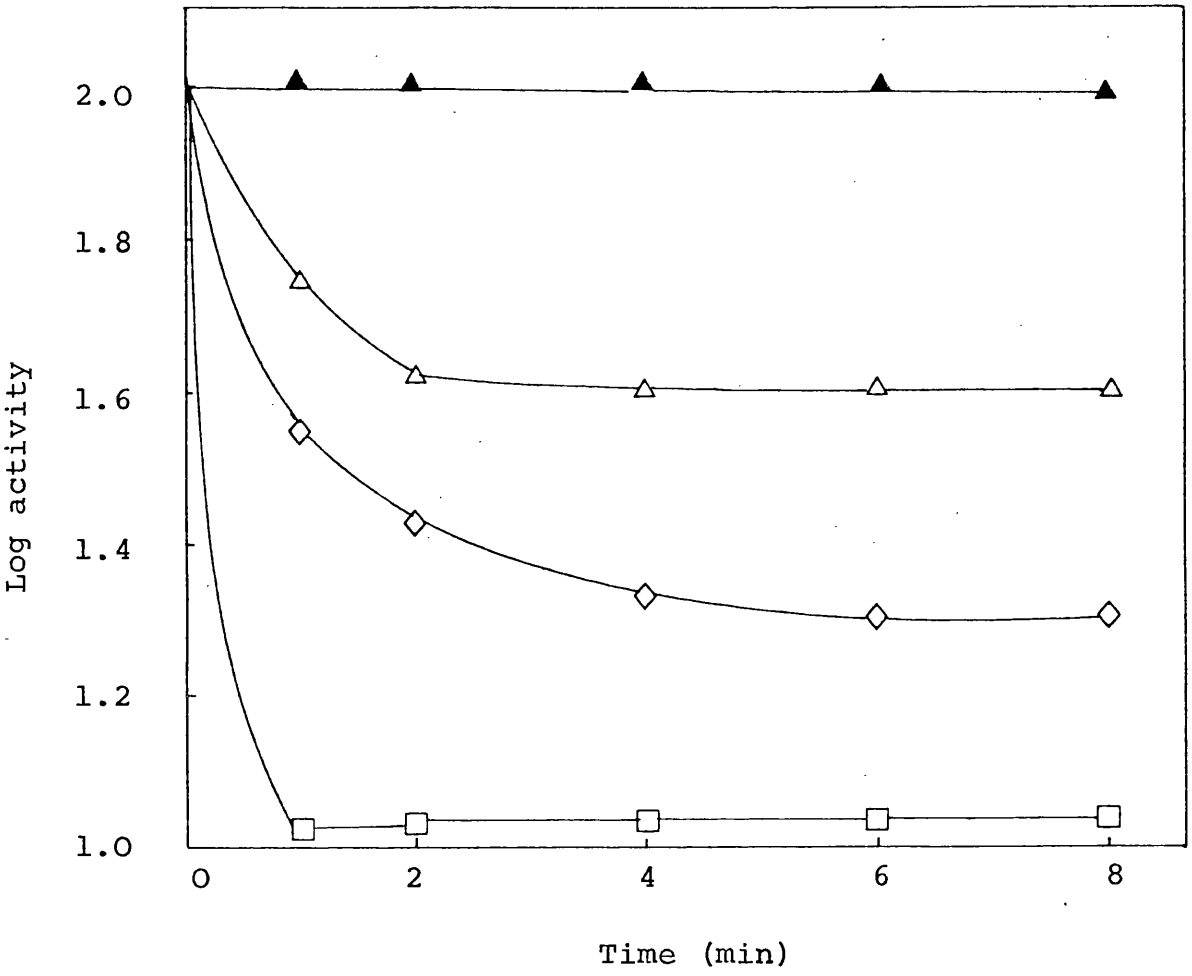


Fig.59. Effect of salt on the modification of E.coli citrate synthase by DTNB
Enzyme was incubated at 20°C with 0.5 mM-DTNB in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme assayed in the absence of 0.1 M-KCl;
(▲) Enzyme assayed in the presence of 0.1 M-KCl;
(◇) Enzyme modified in the presence of 0.1 M-KCl;
(□) Enzyme modified in the presence of 10 mM-$MgCl_2$.

Wild-type Ps.aeruginosa citrate synthase was far more sensitive to inactivation by 4-PDS (10 μM) and 2-PDS (10 μM) than by DTNP (0.2 mM) (Fig.60) or DTNB (0.1 mM) (Fig.53). In all cases, salt did not stimulate the rate of inactivation, in fact it appeared to afford some protection. Mutant Ps.aeruginosa CS I was similarly affected (Fig.61). None of these reagents inactivated mutant Ps.aeruginosa CS II even at a concentration of 0.5 mM in the absence or presence of salt.

E.coli citrate synthase was not inactivated by 4-PDS or 2-PDS unless salt was present (Fig.62). The enzyme was far less sensitive to these reagents than were the Pseudomonas enzymes. A.calcoaceticus citrate synthase was not affected by DTNB but was inactivated by 4-PDS, 2-PDS and DTNP, and this inactivation was enhanced by salt (Fig.63).

Pig heart citrate synthase was slowly inactivated by 4-PDS in the presence of 0.1 M-KCl and by 2-PDS in the presence or absence of 0.1 M-KCl (Fig.64). No inactivation was seen with DTNB. These results showed that diverse citrate synthases respond in different ways to a variety of thiol-blocking reagents.

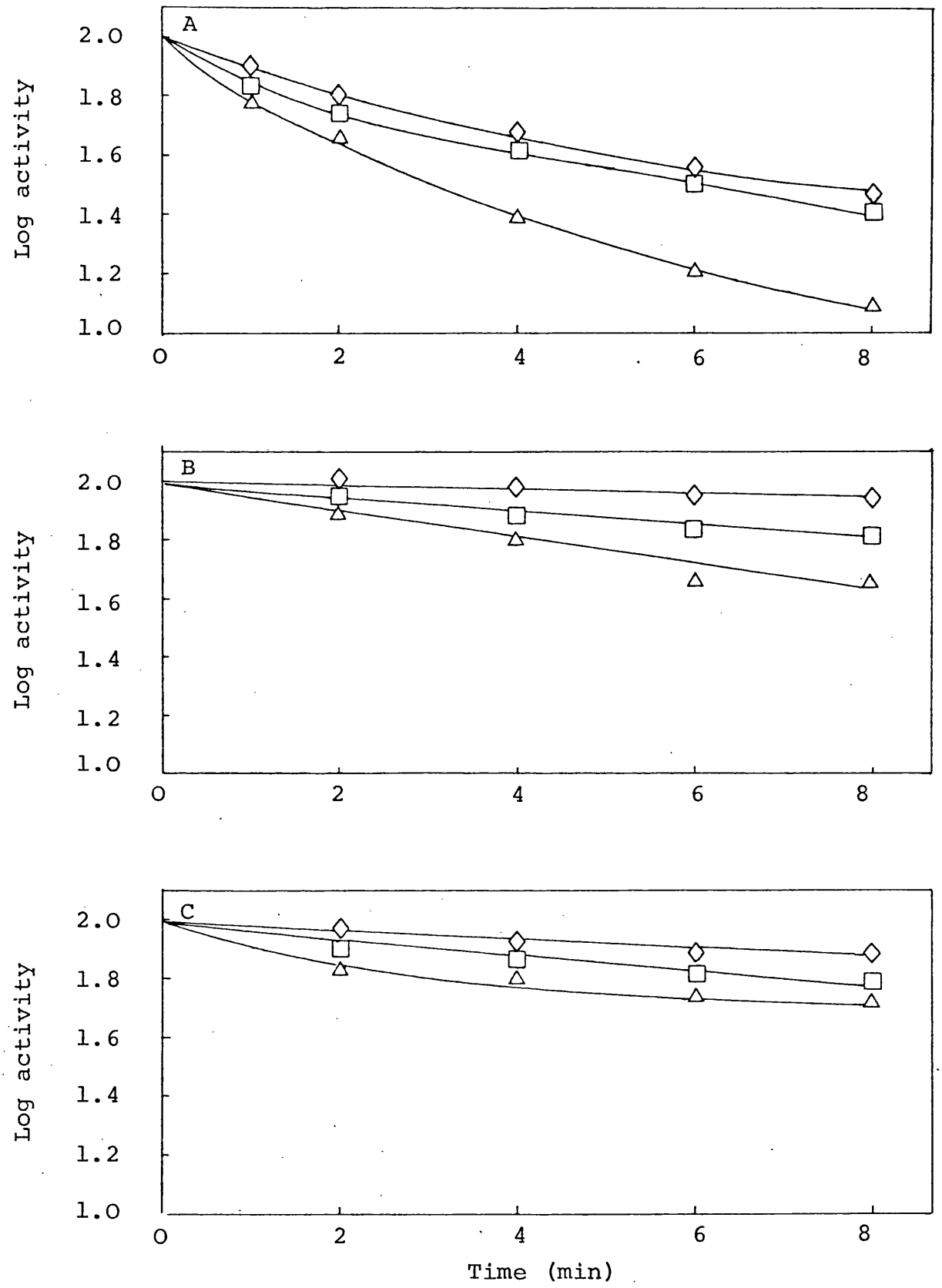


Fig.60. Comparison of the effects of 4-PDS, 2-PDS and DTNP on wild-type Ps.aeruginosa citrate synthase

Enzyme was incubated at 20°C with 10 µM-4-PDS (curve A), 10 µM-2-PDS (curve B) and 0.2 mM-DTNP (curve C) in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme modified in the absence of salt;
(◇) Enzyme modified in the presence of 0.1 M-KCl;
(□) Enzyme modified in the presence of 10 mM-$MgCl_2$.

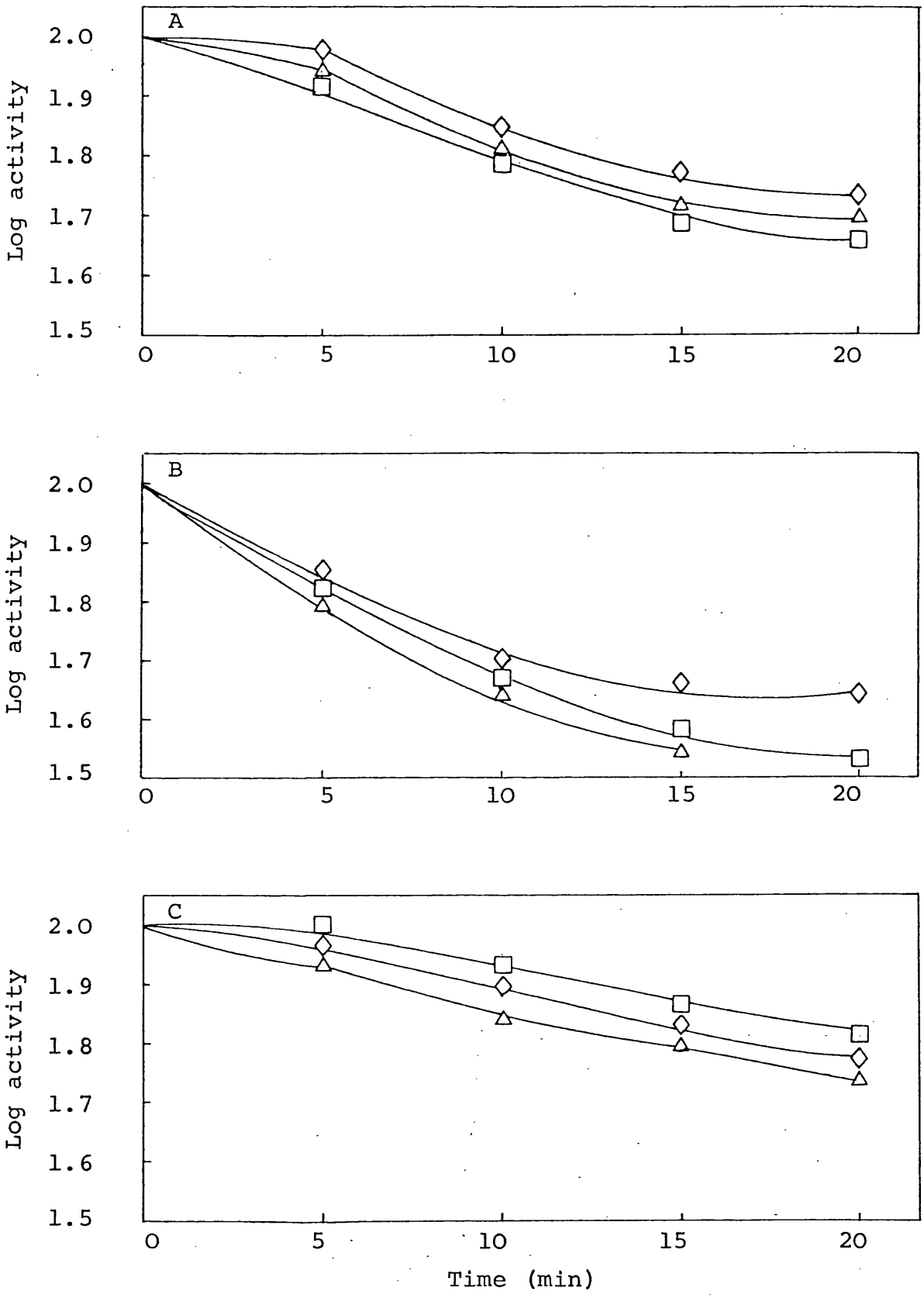


Fig.61. Comparison of the effects of 4-PDS, 2-PDS and DTNP on mutant Ps.aeruginosa CS I

Enzyme was incubated at 20°C with 5 µM-4-PDS (curve A), 5 µM-2-PDS (curve B) and 0.2 mM-DTNP (curve C) in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme modified in the absence of salt;
(◇) Enzyme modified in the presence of 0.1 M-KCl;
(□) Enzyme modified in the presence of 10 mM-$MgCl_2$.

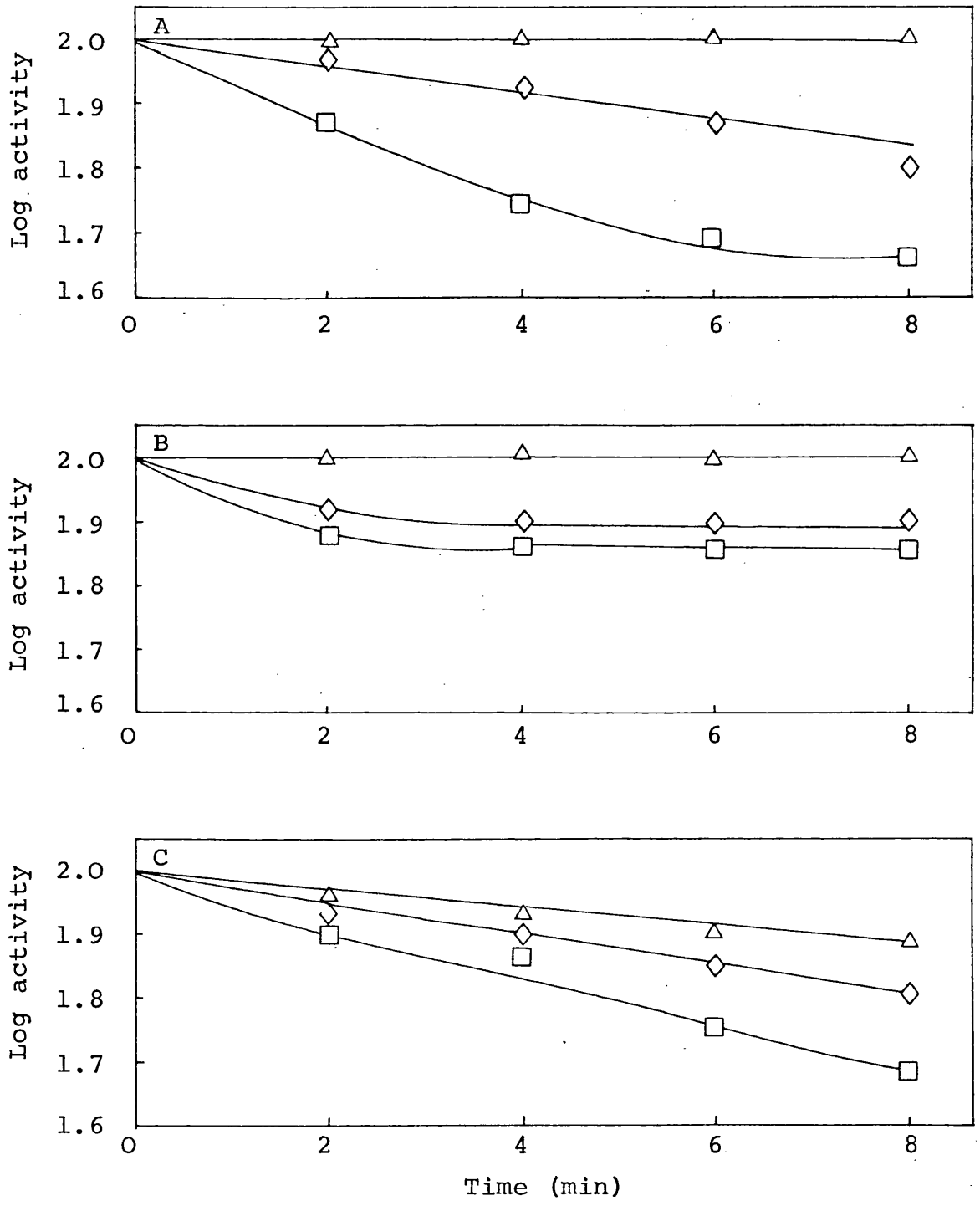


Fig.62. Comparison of the effects of 4-PDS, 2-PDS and DTNP on E.coli citrate synthase

Enzyme was incubated at 20°C with 0.1 mM-4-PDS (curve A), 0.1 mM-2-PDS (curve B) and 0.2 mM-DTNP (curve C) in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme modified in the absence of salt;
(◇) Enzyme modified in the presence of 0.1 M-KCl;
(□) Enzyme modified in the presence of 10 mM-$MgCl_2$.

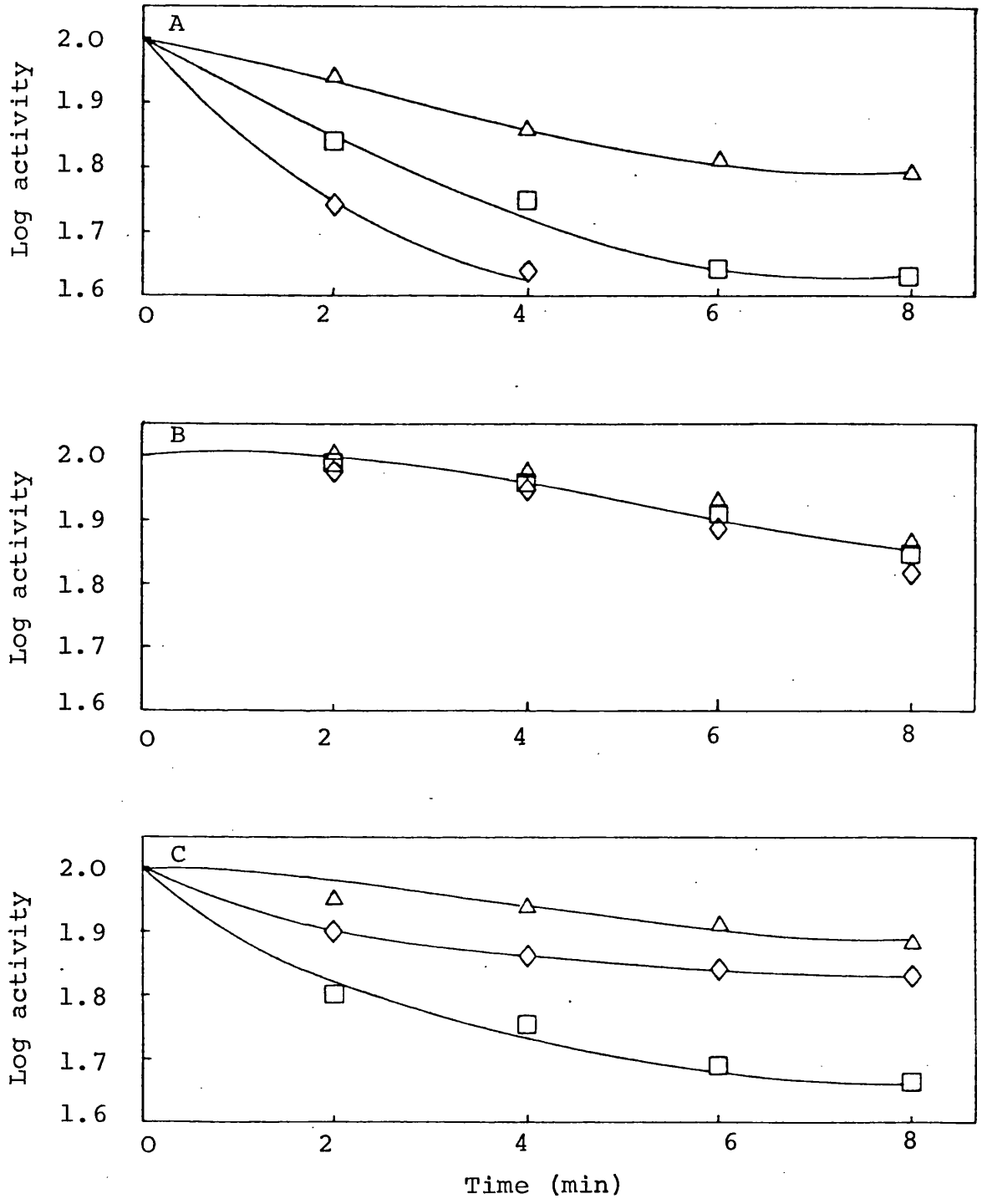


Fig.63. Comparison of the effects of 4-PDS, 2-PDS and DTNP on A.calcoaceticus citrate synthase

Enzyme was incubated at 20°C with 0.1 mM-4-PDS (curve A), 0.1 mM-2-PDS (curve B) and 0.2 mM-DTNP (curve C) in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme modified in the absence of salt;
(◇) Enzyme modified in the presence of 0.1 M-KCl;
(□) Enzyme modified in the presence of 10 mM-$MgCl_2$.

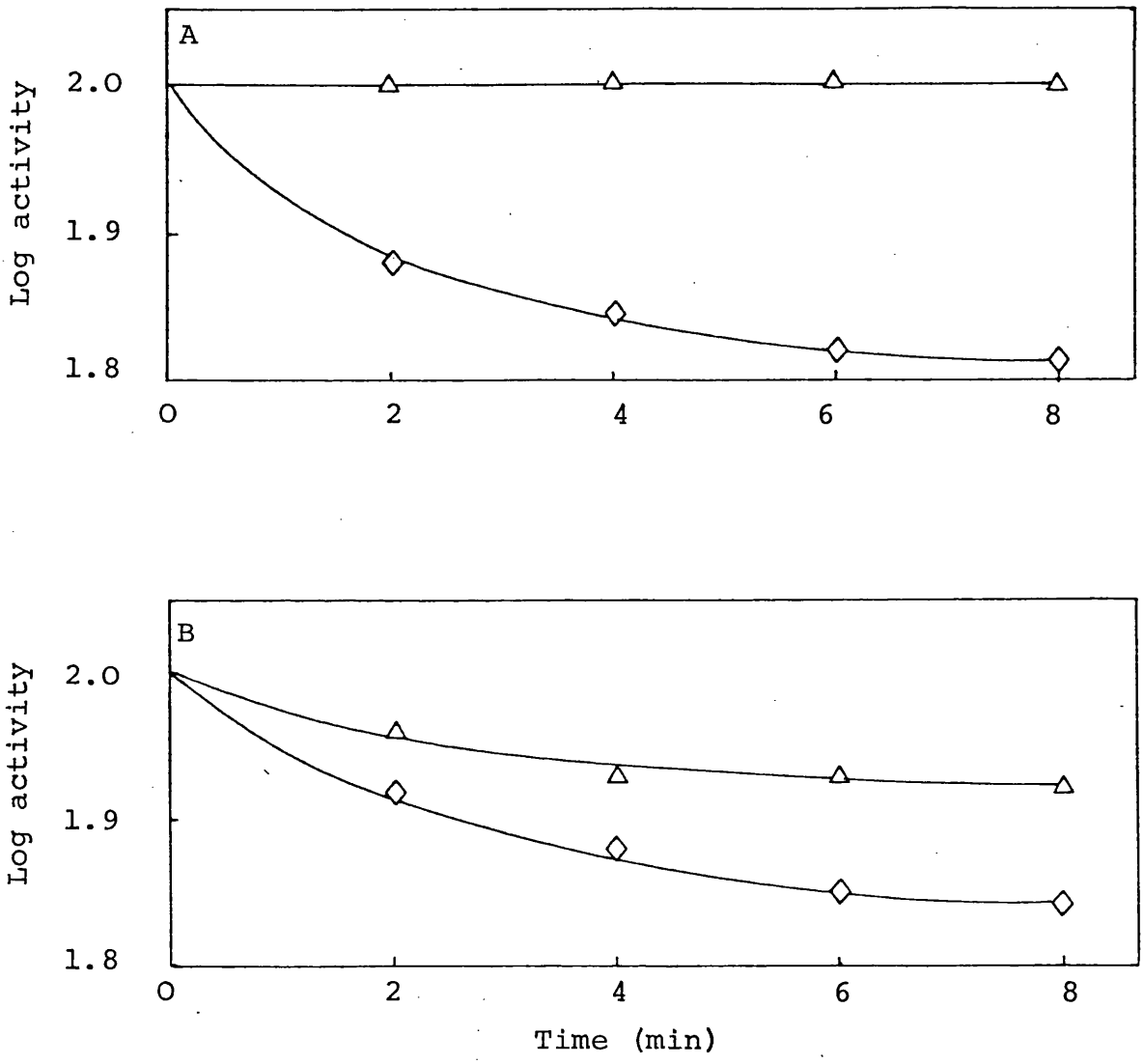


Fig.64. Comparison of the effects of 4-PDS and 2-PDS on pig heart citrate synthase
Enzyme was incubated at 20°C with 0.1 mM-4-PDS (curve A) and 0.1 mM-2-PDS (curve B) in 'Tris buffer (pH 8.0)'.
Enzyme activity was measured using assay Method 1.
(△) Enzyme modified in the absence of salt;
(◇) Enzyme modified in the presence of 0.1 M-KCl.

DISCUSSION

Unity and Diversity of Citrate Synthase

The division of bacterial citrate synthases into two distinct groups - 'large' NADH-sensitive and 'small' NADH-insensitive - was quite unexpectedly found to correlate with the taxonomic division of bacteria based on their response to the Gram stain (Weitzman and Jones, 1968). Gram-negative bacteria were found to contain the 'large' enzyme and Gram-positive bacteria the 'small' enzyme. The Gram-positive enzyme resembled the citrate synthases of eukaryotic organisms (Table 11).

This division between 'large' and 'small' enzymes was so sharp that it has provided the basis of a method used successfully to assist the classification of some bacteria whose taxonomic status was uncertain (Jones and Weitzman, 1971, 1974).

It is generally believed that different responses to the Gram stain are associated with differences in the molecular architecture of the cell wall (Salton, 1964). In addition to differences in cell wall composition, the two groups of bacteria exhibit differences in cell membrane structure. The Gram-positive bacteria show greater structural complexity and the distinction between cell wall and membrane is far more pronounced than in

Table 11

Correlation between the regulation of citrate synthase and the taxonomic status of the organism

GRAM-NEGATIVE PROKARYOTES		GRAM-POSITIVE PROKARYOTES AND EUKARYOTES
NADH inhibition (Allosteric)		No NADH inhibition ATP inhibition (Isosteric)
Obligate aerobes	Facultative anaerobes	
AMP overcomes NADH inhibition	No AMP effect	
No α-Oxoglutarate inhibition	α-Oxoglutarate inhibition (Allosteric)	

Gram-negative bacteria (Rogers and Perkins, 1968); it is therefore plausible that differences in structural organization between Gram-negative and Gram-positive bacteria could give rise to different metabolic 'lifestyles' and result in differences in the regulation of citrate synthase as well as differences in other enzymes.

Indeed, Weitzman and Kinghorn (1978) have shown that succinyl-CoA synthetases occur in either tetrameric or dimeric forms and the division coincides with the findings on citrate synthase, i.e. only 'large' succinyl-CoA synthetases are found in Gram-negative bacteria whereas 'small' succinyl-CoA synthetases are associated with Gram-positive bacteria and eukaryotic organisms. Ball and Nishimura (1980) have shown that rat heart succinyl-CoA synthetase consists of an $\alpha\beta$ dimeric subunit structure. SDS-polyacrylamide-gel electrophoresis revealed a molecular weight of 46,500 for the β subunit and 33,500 for the α subunit. Pig heart citrate synthase has been shown to consist of an α_2 dimeric structure of subunit molecular weight 48,000 (Singh et al., 1970). It has been suggested by Weitzman and Kinghorn (1978) that as both citrate synthase and succinyl-CoA synthetase utilize an acyl-CoA

as substrate, and Gram-negative citrate synthases have a distinct (regulatory) nucleotide binding site while succinyl-CoA synthetase has a catalytic nucleotide binding site, there may be some evolutionary link between the two enzymes. The similarity in the molecular weight of the β subunit of succinyl-CoA and the subunits of citrate synthase may support this possibility.

Henderson et al. (1979) have shown that the E2 structural core of the pyruvate dehydrogenase multi-enzyme complex of B.stearothermophilus, a Gram-positive organism, closely resembles the E2 structural core of the mammalian complex. They both contain 60 subunits and exhibit icosahedral symmetry, whereas the E2 structural core of the Gram-negative E.coli complex contains only 24 subunits and exhibits octahedral symmetry. This correlates precisely with the division of citrate synthase and succinyl-CoA synthetase types between Gram-negative bacteria and Gram-positive bacteria and eukaryotes.

Malate dehydrogenase has also been shown to occur in 'large' and 'small' forms, the molecular weight of the 'large' form being twice that of the 'small' enzyme

(Murphey et al., 1967). The occurrence of the 'large' malate dehydrogenase was restricted to certain Gram-positive bacteria, particularly Bacillus species. This pattern of malate dehydrogenase occurrence does not follow the division between Gram-negative and Gram-positive bacteria exhibited by citrate synthase and succinyl-CoA synthetase. There appears to be no correlation between molecular size patterns for any of the other citric acid cycle enzymes.

Professor Weitzman's research group has intensively investigated the structure-function relationships in the two categories of citrate synthase. One of the outstanding problems is to decide how closely related the structural genes in diverse organisms are to one another and whether 'large' and 'small' citrate synthases have a common subunit.

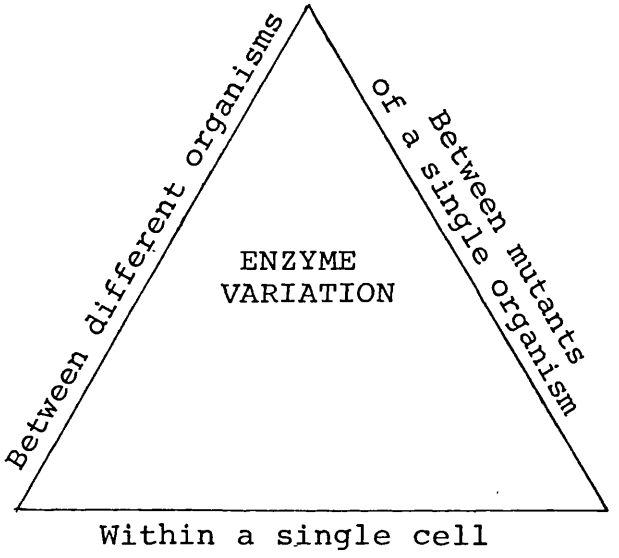
The generation of various mutant bacterial citrate synthases has been particularly useful in this approach. Harford and Weitzman (1978) generated 'large' and 'small' citrate synthases by selection of spontaneous revertants of citrate synthase-deficient strains of E.coli. The citrate synthase-deficient strains were obtained by mutation with ethylmethanesulphonate and were selected

as glutamate auxotrophs when grown on glucose or succinate. Danson et al. (1979) showed by a series of tests such as Gram-staining, electron micrographic examination of cell wall structure and by the conservation of genetic markers from the wild-type to the mutant that the mutant was indeed a strain of E.coli.

There was still a possibility that the 'small' enzyme was a product of a different gene from that normally coding for the typical 'large' E.coli enzyme. The mutation might have arisen from the inability of the latter to be translated into active enzyme with the switching on of an alternative gene for the 'small' citrate synthase. Gene mapping by conjugation and Phage (P1)-mediated transduction experiments showed that the locations of the citrate synthase gene (gltA) in the wild-type and mutant strains were very close to one another and supported the conclusion that the gene responsible for the production of the 'small' citrate synthase is indeed a modified form of the gene coding for the 'large' enzyme in the wild-type strain.

The remarkable finding that a mutant Ps.aeruginosa contains both a 'large' and 'small' citrate synthase

provided an excellent opportunity to examine further the structure-function relationships of citrate synthase and completed a three-sided investigative approach currently being used by Weitzman and co-workers.



One other instance of two forms of citrate synthase in the same organism has been reported. Massarini and Cazzulo (1975) isolated a 'large' NADH-sensitive and a 'small' NADH-insensitive citrate synthase from a marine pseudomonad. The 'small' form of the enzyme could be obtained by dissociation of the 'large' enzyme by dialysis against phosphate buffer (pH 7.0) and removal of the inorganic phosphate by dialysis resulted in reassociation of the 'large' enzyme (Higa et al., 1978). The evidence of Higa et al. (1978) suggests that in this particular organism the 'large' form of the enzyme exists in vivo and that dissociation occurs in vitro.

Growth-Phase Dependent Citrate Synthase Variation in Ps.aeruginosa

The variation of the levels of CS I (a 'large' citrate synthase) and CS II (a 'small' citrate synthase) in the mutant Ps.aeruginosa described in this thesis is the first report of growth-dependent enzyme variation within the citrate synthase system. The mutant was one of a series isolated by Skinner and Clarke (1968) after treatment of Ps.aeruginosa 8602 with the chemical mutagens ethylmethanesulphonate and N-methyl-N'-nitro-N-nitrosoguanidine. Ps.aeruginosa produces an inducible amidase in media containing acetamide and this method was used to obtain mutants lacking amidase activity. However it was found that those mutants unable to grow on acetamide or acetate still contained amidase, indicating that the metabolic defect must have occurred at another site. Microbial growth on acetate involves the reactions of the glyoxylate cycle (Kornberg and Krebs, 1957) and indeed it was found that several of these mutants had defects in isocitrate lyase and malate synthase. The mutant At 14 was found to have a normal malate synthase activity and a less than normal isocitrate lyase activity when grown on succinate.

At 14 was the only mutant to have a very low citrate synthase activity (7% of the wild-type). A low citrate

synthase activity could result in an increased intracellular concentration of oxaloacetate and consequently of phosphoenolpyruvate which is thought to be the specific co-repressor of isocitrate lyase in E.coli (Kornberg, 1966). It was therefore concluded that the acetate-negative character of mutant At 14 was partly due to the consequent phenotypic deficiencies of isocitrate lyase. Preliminary investigations of mutant At 14 in this laboratory showed a 'small' citrate synthase to be present in very low levels. However, subsequent investigations showed that two forms of citrate synthase were present when the organism was grown on nutrient-glutamate. CS I was found to predominate in a log-phase culture, whereas when the culture reached stationary phase the proportion of CS II increased until it became the predominant form. The level of CS II in a stationary-phase culture was as high as that of citrate synthase in the wild-type Ps.aeruginosa at a similar stage of growth. The mutant pseudomonad is therefore not a citrate synthase-deficient strain.

One possibility for the change in the level of CS II was that CS I dissociated, or was proteolytically degraded, to CS I. No dissociation of CS I was observed in vitro unlike the situation described above for the 'large' citrate synthase found in a marine pseudomonad

(Higa et al., 1978). One case of proteolytic degradation of citrate synthase has been reported. Eukaryotic citrate synthases are located in the mitochondria and Harmey and Neupert (1979) have shown that the dimeric Neurospora crassa citrate synthase is synthesized in the cytosol as a large precursor of subunit molecular weight 47,000 whereas the enzyme isolated from the mitochondria has a subunit molecular weight of 45,000. They concluded that a small section of the enzyme is cleaved on transfer of the enzyme from the cytosol to the mitochondria. They were unable to show the precise nature of the cleavage but thought there was likely to be a protease present inside the mitochondria.

Thus it would be possible for CS I to be modified by a specific protease resulting in the formation of CS II. However there was no decrease in the level of CS I from a log-phase culture to a stationary-phase culture. This was also confirmed by treatment of a log-phase culture with chloramphenicol. In the absence of protein synthesis there was no conversion of CS I to CS II unless the chloramphenicol actually prevented the synthesis of the protease. It appears that CS II was a result of de novo protein synthesis.

There was a possibility that two citrate synthase genes were present, one coding for CS I and the other for CS II. As discussed previously, the E.coli mutants were shown to have an altered structural gene and there has been no previous report of two citrate synthase genes in the same organism. This possibility cannot be eliminated until the mapping of the citrate synthase genes in both wild-type and mutant Ps.aeruginosa is carried out.

If, as in the case of the mutant E.coli, there is a minor alteration in the structural gene coding for citrate synthase, one would expect the subunit structure of CS I and CS II to be closely related to one another. Evidence from gel filtration experiments suggest that the molecular weight of CS I is about 300,000 and that of CS II is about 100,000. This would be consistent with the dimeric-hexameric relationship between Gram-positive and Gram-negative bacterial citrate synthases. However, until the two enzymes have been purified to homogeneity and their subunit molecular weights and amino acid compositions determined, the extent of the similarity between the two enzymes cannot be decided.

One remarkable feature about the occurrence of CS I and CS II in a log-phase liquid culture is that

the ratio of the two enzymes varied according to the age of the colony taken from the solid medium. The older the colony, the more CS II was obtained in a log-phase culture. Whatever changes take place in the cell during growth on a liquid culture which alter the levels of CS I and CS II must also take place on solid medium.

Wimpenny (1979) has shown that when bacteria grow on solid media they also exhibit exponential growth for part of the time. Large colonies of Enterobacter cloacae were frozen and horizontal sections of these frozen colonies were made (Wimpenny and Parr, 1979). Crude extracts were obtained by sonication and it was possible to assay enzyme activities in different locations within the colonies. It was found that there was a variation in the activities of some enzymes depending on their location within the colonies. It may be possible to use the same procedure to obtain extracts of mutant Ps.aeruginosa from colonies of different ages and to investigate the variation of CS I and CS II in these colonies.

As cultures grow exponentially the "energy charge", defined as $\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$ remains at a high level

(0.8 - 0.95) (Chapman et al., 1971). Viable cells in stationary phase maintain an energy charge of about 0.6 whereas in cells about to die the value falls to 0.5. These observations have been made in Ps.aeruginosa (Wiebe and Bancroft, 1975). In addition, Wiebe and Bancroft showed that the break between log- and stationary-phase energy charge values preceded the observed change in growth rate. This same 'anticipation' phenomenon was observed when ATP was measured alone or when respiration was examined. It appeared that even before the apparently linear growth ended there was a shift in metabolic function.

It may be that changes in levels of CS I and CS II are associated with a shift in metabolic function. CS I is present during log phase when the cells are actively growing and is regulated by the effectors AMP and NADH whereas the non-regulated form of citrate synthase (CS II) appears as the culture approaches stationary phase. If this shift in metabolic function causes changes in the ratios of CS I to CS II it is still not known what actually switches on the synthesis of CS II.

Other growth-phase dependent enzymes have been found in bacteria. Goodman and Bessman (1973) showed that there were two growth-phase dependent deoxyribonucleic

acid polymerases in Lactobacillus acidophilus. One enzyme had a molecular weight of 78,000 and was found in both log- and stationary-phase cells. The second enzyme had a molecular weight of 92,000; it first appeared in late log phase and increased to 45% of the total enzyme activity in stationary-phase cells. There was very little difference in the catalytic activities of the two enzymes, the major differences being that only the small enzyme was stimulated by spermidine and the larger enzyme showed a preference for native DNA.

Goodman and Bessman (1973) stated that lactobacilli undergo dramatic morphological changes during the growth cycle which are reflected in distinct cell elongations and changes from Gram-positive to Gram variable staining characteristics. They believed the appearance of the second enzyme may be associated with one of these architectural changes in the cell wall and membrane.

Aspartate transcarbamoylases (ATCases) derived from various bacterial species can be grouped into distinct classes (Bethell and Jones, 1969). The 'large' enzyme (ATCase I) had a molecular weight of 300,000 and the 'small' enzyme (ATCase II) had a molecular weight of 100,000. As in the case of citrate synthases, only

the 'large' enzyme was subject to feedback control. ATP activated ATCase I but had little effect on ATCase II whereas CTP inhibited only the 'large' enzyme.

Coleman and Jones (1971) found two ATCases in Citrobacter freundii. The 'large' enzyme could be converted into the 'small' enzyme in vitro by dissociation during gel filtration, which suggested that the two enzymes possess a common subunit. ATCase II was the only form of the enzyme found in a middle log-phase culture whereas ATCase I was the only enzyme form found in a late log-phase culture. Thus in this organism the 'small' enzyme appears to be converted into a larger form during growth of the culture. Coleman and Jones (1971) suggested that pulse-labelling of ATCase II before ATCase I was present would demonstrate whether this is indeed the case, by seeing whether the label is incorporated into ATCase I, but at the present time this experiment has not yet been carried out.

A similar experiment would show whether CS II is derived from CS I in the mutant Ps.aeruginosa. However, the fact that there is no decrease in the level of CS I during growth suggests this to be unlikely.

In addition to the variation in the levels of CS I and CS II observed during growth on nutrient-glutamate,

variations in these enzymes was also studied during growth on single carbon sources. Growth on acetate, aspartate, citrate, fumarate or pyruvate resulted in an elevated level of CS II in log-phase cultures with very little change in the citrate synthase ratios in stationary-phase cultures apart from an increase in CS II on fumarate. Growth on glucose, glutamate, glycerol, malate or succinate resulted in almost identical levels of CS I and CS II in a log-phase culture and a slight increase in the level of CS II in stationary-phase cultures especially on glucose and malate.

There appeared to be no obvious correlation between the nature of the carbon source and the levels of CS I and CS II in a liquid culture of mutant Ps.aeruginosa. There was an elevated level of CS I on nutrient-glutamate in a log-phase culture and this level of enzyme was maintained when the mutant was grown to log phase on resterilized spent medium. It was therefore unlikely that it was the result of a change in the medium which caused an increased level of CS II in a stationary-phase culture.

Transfer of a log-phase culture grown on nutrient-glutamate to acetate medium lacking a supply of nitrogen resulted in an elevated level of CS II with no change in the level of CS I or the total protein. The presence

of acetate in the medium appeared to induce the synthesis of CS II. Transfer of a log-phase culture to carbon-free or carbon- and nitrogen-free medium resulted in no change of citrate synthase levels. Only the nature of the carbon source seemed to influence the levels of citrate synthase.

Other instances of differing enzyme forms being present when bacteria are grown on different carbon sources have also been found. Scamuffa and Caprioli (1980) showed that there were two distinct aldolases (Class I and II) when E.coli was grown on gluconeogenic compounds such as lactate and pyruvate, whereas only a single enzyme (Class II aldolase) was present when growth was on glucose or fructose. Class I aldolases are normally found in higher plants and animals and Class II aldolases in bacteria and fungi (Heron and Caprioli, 1975). Class I aldolases use a Schiff-base mechanism whereas Class II aldolases employ a metal-chelate mechanism. It has been suggested that the E.coli Class I enzyme has a biosynthetic function and the constitutive Class II enzyme a glycolytic function.

Two forms of isocitrate lyase have been found in Yersinia pestis (Hillier and Charnetzky, 1981). Type A resembled the enzyme found in Enterobacteriaceae in that it was induced during growth on acetate and

repressed during growth on glucose or xylose. Type B isocitrate lyase was found during growth on acetate, glucose, xylose and various amino acids. Type A isocitrate lyase serves an anaplerotic function but, as yet, the role of Type B isocitrate lyase is unknown.

Purification of Wild-Type and Mutant Ps.aeruginosa Citrate Synthases

In order to decide how closely related CS I and CS II are to one another it is necessary to investigate the molecular and regulatory properties of the two enzymes. This can only be achieved if sufficient quantities of pure enzyme are obtained. This same problem is found with all the mutant bacterial citrate synthases. Conventional purification procedures take several days and involve a number of different steps; consequently there is not a very high recovery of the mutant enzymes which are usually present in low levels and are rather unstable. For this reason several affinity ligands were prepared in order to improve the recovery of the enzyme and reduce the number of steps involved in the purification procedure.

A successful affinity chromatography enzyme separation involves the covalent attachment of a bio-specific ligand to a chromatographic bed material,

the matrix. It is also important that the immobilized ligand retains its specific binding affinity for the enzyme and that methods are available for selectively desorbing the bound enzyme in an active form, after washing away unbound material. Suitable ligands for enzymes can be substrates, cofactors or effectors, or analogues of these compounds.

Sepharose 4B is the most favoured and widely-used matrix and was used in these studies. Sepharose 4B is a bead-formed agarose gel whose hydroxyl groups on the sugar residues can easily be derivatized for covalent attachment of the ligand.

The selection of the ligand for affinity chromatography is influenced by two factors. First, the ligand should exhibit specific and reversible binding affinity for the enzyme to be purified. Secondly, it should have chemically modifiable groups which allow it to be attached to the matrix without destroying its binding activity.

The active site of an enzyme may be located deep within the molecule and by binding the ligand directly to the matrix there may be some steric hindrance between the enzyme and the matrix. This steric hindrance can be overcome by inserting a spacer group between the matrix and the ligand. In the ligands prepared for citrate

synthase a 6-carbon spacer was chosen. The length of the spacer arm is critical. If it is too short it is ineffective and if it is too long non-specific effects such as hydrophobic interactions may be exerted (O'Carra et al., 1973).

CoASH was initially chosen as the ligand for citrate synthase. It is a large molecule and there are a number of possible sites for covalent attachment to the matrix (Fig.65). Attachment of CoASH through the thiol group at position A to Activated Thiol-Sepharose and of dephospho-CoASH through the vicinal 2'-3' hydroxyl groups of the ribose at position B resulted in no retention of citrate synthase. These ligands were therefore of no potential value for the purification of citrate synthases, presumably because the groups modified are essential for enzyme recognition.

Attachment of CoASH to Sepharose 4B through the 6-amino group of the adenine residue at position C using either the CNBr-activation method or the carbodiimide method in the presence of a spacer resulted in very powerful binding of citrate synthase but the enzyme could not be recovered. Similar effects were seen when the CoASH ligand was acetylated. It is vital that the ligand-enzyme interaction is reversible for a successful purification. The use of Activated CH-Sepharose

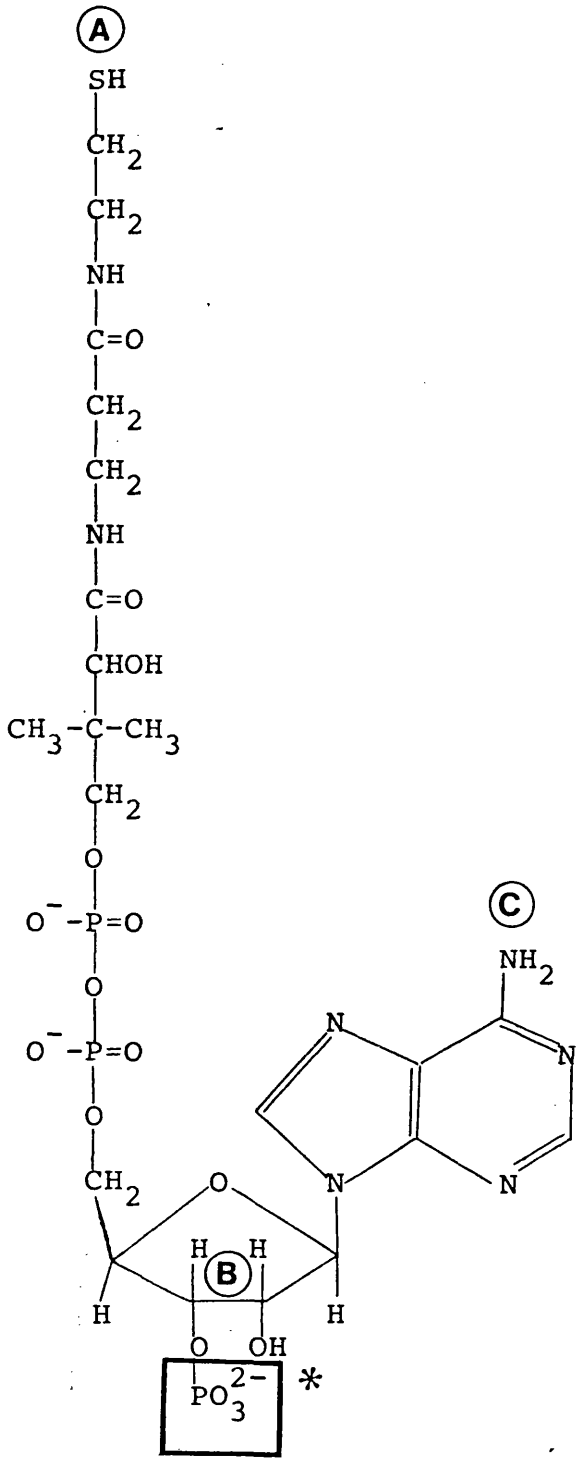


Fig.65. Sites for covalent attachment of CoASH to Sepharose

(A) Attachment to Activated-Thiol Sepharose

(B) Attachment to Sepharose-adipic acid hydrazide

(C) Attachment to CNBr-activated Sepharose, CH-Sepharose and Activated CH-Sepharose

* dephospho-CoASH

PO_3^{2-} replaced by H

enabled acetyl-CoA to be bound directly; however, the binding between ligand and enzyme was again very powerful.

The use of oxaloacetate and other intermediates of the citric acid cycle as ligands also resulted in very little purification of citrate synthase. These molecules are much smaller than CoASH and contain a number of reactive carboxyl groups. The binding of the enzyme to these immobilized ligands was probably through a non-specific ion-exchange effect.

A number of group-specific ligands (AMP, ADP, ATP and NADH) were also prepared. Group-specific ligands have an affinity for a group of related substances rather than for a single substance. The same general ligand can therefore be used to purify several enzymes without the requirement that a new ligand be prepared for each enzyme to be purified. However, in order to obtain a successful purification a specific elution is required. Mukherjee and Srere (1976) as well as studies in this laboratory have shown that a combination of 0.1 mM-CoASH and 0.1 mM-oxaloacetate is effective in eluting citrate synthases ('large' and 'small') from group-specific ligands. However, none of the group-specific ligands prepared was found to be effective in the purification of the mutant citrate synthases. Matrex

Gel Red A can be considered a group-specific ligand which exhibits a high affinity for NADP-requiring enzymes. Matrex Gel Red A consists of the dye Procion Red HE3B covalently attached to 5% crosslinked agarose and has been used with considerable success in the purification of A.calcoaceticus citrate synthase (C.G. Mitchell, personal communication).

Matrex Gel Red A was the only group-specific ligand which was successfully used in the purification of CS I and CS II. CS I could not be eluted from Matrex Gel Red A by a mixture of 0.1 mM-CoASH and 0.1 mM-oxaloacetate, whereas CS II could be so eluted. This illustrated that there is variation in the properties of the different types of citrate synthase and this makes the search for a ligand which can be used for all citrate synthases extremely difficult. It is largely a question of trial and error in choosing the correct ligand, method of attachment and conditions of elution.

Kinetic and Regulatory Properties of Wild-Type and Mutant Ps.aeruginosa Citrate Synthases

Though CS I and CS II have not as yet been purified to homogeneity it was still possible to investigate some of their properties and compare them with those of the wild-type Ps.aeruginosa citrate synthase.

Gel filtration studies showed the molecular weight of CS I to be similar to that of the wild-type enzyme (≈300,000) and consistent with its classification as a 'large' enzyme. However, this was only an estimation and a more precise determination using techniques such as analytical ultracentrifugation of the native enzyme and SDS-polyacrylamide-gel electrophoresis of the subunits is required. Such studies will have to await the availability of pure enzyme. CS II was shown to be of the 'small' type with a molecular weight of about 100,000.

The wild-type enzyme was shown to have a sigmoidal rate dependence on acetyl-CoA concentration and a hyperbolic dependence on the second substrate, oxaloacetate. Additionally, assays in the presence of 0.1 M-KCl revealed a hyperbolic rate dependence on acetyl-CoA concentration. It was likely that KCl induced a conformational change in the enzyme which abolishes the cooperativity between the subunits. This was similar to the dependences seen with citrate synthase of E.coli, a facultative anaerobe, whereas the citrate synthases of aerobic bacteria have been thought to exhibit hyperbolic rate dependences for both substrates (Weitzman and Danson, 1976). Ps.aeruginosa citrate synthase does not fit into this general pattern described above and illustrates that even though citrate synthases can be classified

into 'large' and 'small' and, in Gram-negative aerobic bacteria, are deinhibited by AMP in the presence of NADH, there are still differences between enzymes within a particular category. Morse and Duckworth (1980), using the statistical method of Cornish-Bowden (1978), have examined the sequence homologies from the amino acid compositions of citrate synthases from E.coli, A.calcoaceticus and Ps.aeruginosa. They estimated the sequence homology of the amino acid sequences of the E.coli and A.calcoaceticus enzymes to be 92%, whereas preliminary investigations of Ps.aeruginosa citrate synthase indicated about 69% homology with the E.coli enzyme and 65% homology with the A.calcoaceticus enzyme. Even though both A.calcoaceticus and Ps.aeruginosa citrate synthases are classified in the same category, there is greater homology between the A.calcoaceticus and E.coli enzymes.

The wild-type Ps.aeruginosa citrate synthase had a sigmoidal dependence of NADH inhibition on NADH concentration which was abolished by 0.1 M-KCl and by modification with thiol-blocking reagents. This provided evidence that the NADH binding site is allosteric. Other nucleotides had very little effect on activity and probably exerted a non-specific isosteric effect (as did NADH on the desensitized enzyme) resulting from the common structural features shared by nucleotides and the substrate acetyl-CoA.

Multiple-inhibition studies by Harford and Weitzman (1975) showed that there are allosteric and isosteric sites in the Ps.aeruginosa enzyme and that the non-specific nucleotide inhibitors are indeed competitive with acetyl-CoA.

AMP overcame the inhibition by NADH of the wild-type enzyme but had no effect on the enzyme in the absence of NADH. Rowe and Weitzman (1969) presented evidence from electron microscopy studies that conformational changes are associated with the binding of NADH and AMP to A.calcoaceticus citrate synthase. Measurements of particle or shadow widths indicated a swelling of the molecule in the presence of NADH and a reversal of this enlargement by either the deinhibitor AMP or the desensitizer KCl. Swellings were not seen with either NAD^+ or NADPH, indicating the specificity of the inhibition. Analytical ultracentrifugation studies revealed a 7% retardation in the sedimentation rate of the enzyme in the presence of NADH. In the presence of both NADH and AMP no such retardation was observed. These changes are clearly consistent with an NADH-induced swelling of the enzyme to a less dense, inactive form and an AMP-induced reversal to the more compact, denser, active form.

Results from thermal inactivation studies on the wild-type Ps.aeruginosa enzyme tend to support the view of conformational changes induced by regulatory effectors. Both substrates and the effectors NADH and AMP protected the enzyme from thermal inactivation as did KCl, whereas other nucleotides did not protect the enzyme. No protection of desensitized enzyme was observed by the effectors. As yet there has been no evidence to show whether AMP and NADH bind at the same site or whether AMP binds to the enzyme in the absence of NADH. Protection against thermal inactivation by AMP could be due to binding to a specific site or could result from the structural similarity between AMP and NADH. Citri (1973) has shown the value of thermal inactivation studies in assessing conformational changes of regulatory enzymes.

CS I exhibited a sigmoidal rate dependence on both substrates and a hyperbolic dependence of NADH inhibition on NADH concentration. 0.1 M-KCl did not alter the sigmoidal dependence on the substrates nor did it produce desensitization of the enzyme to NADH inhibition. Thermal inactivation studies showed the enzyme to be protected by 0.1 M-KCl, both substrates, and NADH or AMP. There was no protection by NAD^+, NADPH, $NADP^+$ or ATP. The specificity of the nucleotide inhibition was therefore

conserved in the mutant even though it may have lost its ability to have its conformation altered by KCl. The enzyme was not desensitized to NADH inhibition by DTNB. AMP activated the enzyme in the presence or absence of NADH and the dependence on AMP was of a hyperbolic nature. No AMP deinhibition of NADH-inhibited enzyme was observed. This suggested for the first time that AMP and NADH have separate binding sites on the citrate synthase molecule.

CS II exhibited a hyperbolic rate dependence on both substrates and had properties typical of a 'small' citrate synthase. CS II did not show cooperativity between subunits and was not sensitive to the effectors AMP and NADH; consequently, only the substrates and KCl protected it from thermal inactivation. This was further confirmation that only the larger, more complex, citrate synthases are sensitive to feedback inhibition by NADH.

Chemical Modification of Wild-Type and Mutant *Ps.aeruginosa* Citrate Synthases

It is the aim of enzymology to explain the molecular basis of enzyme function in terms of the particular architecture of the enzyme molecule. One of the methods used is to effect chemical modification of particular residues within the protein and then examine any changes in behaviour in order to assign functional involvement

to particular components of the enzyme. The limitation of this method is that it relies on the specific interaction between the modifying agent and a particular amino acid; any non-specificity will prevent the definite and unambiguous implication of particular amino acids in enzyme function. At the present time, suitable modification methods are not available for all of the amino acids found in proteins. Therefore the role of unreactive amino acids cannot readily be established.

The utility of chemical modification is greatly enhanced by its use in conjunction with sophisticated physical methods which detect fine structural details of proteins. X-ray analysis and N.M.R. studies along with chemical modification can contribute to an understanding of the catalytic mechanism of a particular enzyme.

(a) Photo-oxidation

One of the modification techniques used in the investigation of the catalytic and regulatory properties of citrate synthase is photo-oxidation (Danson and Weitzman, 1973; Weitzman et al., 1974). This involves the interaction between the light-excited molecules of a photo-sensitive dye and ground-state oxygen; the 'activated' oxygen then reacts with an oxidisable substrate. There are five photo-oxidisable amino acids

found in proteins: histidine, tryptophan, tryosine, methionine and cysteine (Weil and Maher, 1950; Weil et al., 1951). Of these five residues cysteine, histidine and tryptophan have been implicated in the catalytic and regulatory properties of citrate synthase (Weitzman and Danson, 1976).

Two photo-sensitive dyes were employed in the photo-oxidation of citrate synthase. These were the cationic dye Methylene Blue and the anionic dye Rose Bengal. The technique involved the illumination with visible light of a mixture of the dye, oxygen and the enzyme. Modification of the enzyme was followed as a function of the time of illumination.

Once the enzyme is modified it is necessary to identify the types of residues photo-oxidised. One way would be to carry out amino acid analyses on enzyme photo-oxidised for different time periods and then identify the residues from the decay rate constants.

Another way is to determine the pH dependences of the photo-inactivations and desensitizations. Weil (1965) has shown that the rate of photo-oxidation of each amino acid depends on the pH of the illuminated reaction mixture. Using this technique Danson and Weitzman (1973)

were able to show that E.coli citrate synthase requires histidine residues for activity and α-oxoglutarate inhibition, and cysteine residues for NADH inhibition. Histidine residues have also been implicated in the activity of A.calcoaceticus citrate synthase (Weitzman et al., 1974).

In the present studies photo-oxidation was used as a probe to investigate differential rates of loss of activity and loss of NADH and AMP regulation. Wild-type and mutant Ps.aeruginosa citrate synthases were not pure enough, nor available in sufficient quantities, to enable an extensive investigation and consequently particular residues could not be assigned specific functions.

Rose Bengal photo-inactivated the Pseudomonas citrate synthases. Oxaloacetate protected the enzymes from inactivation, though the extent of protection with CS I was far less than with the other enzymes. The protection by oxaloacetate could have been due to the fact that the photo-oxidisable residues were involved in the binding of the substrate and therefore in the presence of oxaloacetate the enzymes were no longer photo-inactivated. Wild-type and mutant CS I were also desensitized to NADH inhibition at a different rate to the loss of activity which was consistent with the NADH binding site being allosteric.

The most significant finding was that the loss of NADH inhibition of CS I in the presence of Methylene Blue was far greater than the loss of AMP activation, providing evidence for distinct AMP and NADH binding sites. Previously it had not been possible to show whether AMP and NADH have separate binding sites in citrate synthases of aerobic Gram-negative bacteria because the AMP deinhibition was only observed in the presence of NADH. Desensitization to NADH inhibition by chemical modification prevented any observation of the effect of AMP. Mutation of the wild-type Ps.aeruginosa citrate synthase resulted in the formation of CS I which has lost the AMP deinhibition of NADH-inhibited enzyme, but was activated by AMP in the absence of NADH. It is unlikely that the mutation has resulted in the formation of an additional regulatory site, and more likely that there has been some sort of loss of interaction between the NADH and AMP binding sites. This could be an example of the mutation of a wild-type enzyme enabling the mechanism of its regulation to be explored.

(b) Treatment with specific thiol-blocking reagents

It has been shown that cysteine residues are implicated in the catalytic and regulatory properties of citrate synthase (Weitzman and Danson, 1976). The observation that the wild-type Ps.aeruginosa citrate

synthase was readily desensitized to NADH inhibition at pH 8.0 may have been due to the oxidation of cysteine residues and this prompted an investigation of the role of these residues using specific thiol-blocking reagents.

The reagents used were chromogenic disulphides and reacted by a thiol-disulphide interchange (Brocklehurst, 1979). DTNB was the first specific disulphide to be made (Ellman, 1959) and has been used extensively for the quantitative determination of thiol groups. DTNB reacts with protein thiol groups to form a mixed disulphide and liberates 1 mole of thionitrobenzoate (TNB^-) per mole of modified thiol (Fig.66). The TNB^- anion absorbs strongly at 412 nm and this enables the extent of modification to be monitored and quantitated spectrophotometrically. The carboxyl groups of DTNB confer solubility in aqueous solutions and the electron-attracting nitro groups facilitate the cleavage of its disulphide bond by the attacking nucleophilic thiol anion. As the thiol groups are only reactive in the S^- form the reaction is carried out in mildly alkaline solution (pH 8.0).

Treatment of the wild-type Ps.aeruginosa citrate synthase with DTNB resulted in a slow loss of activity (which was prevented by oxaloacetate) and a rapid

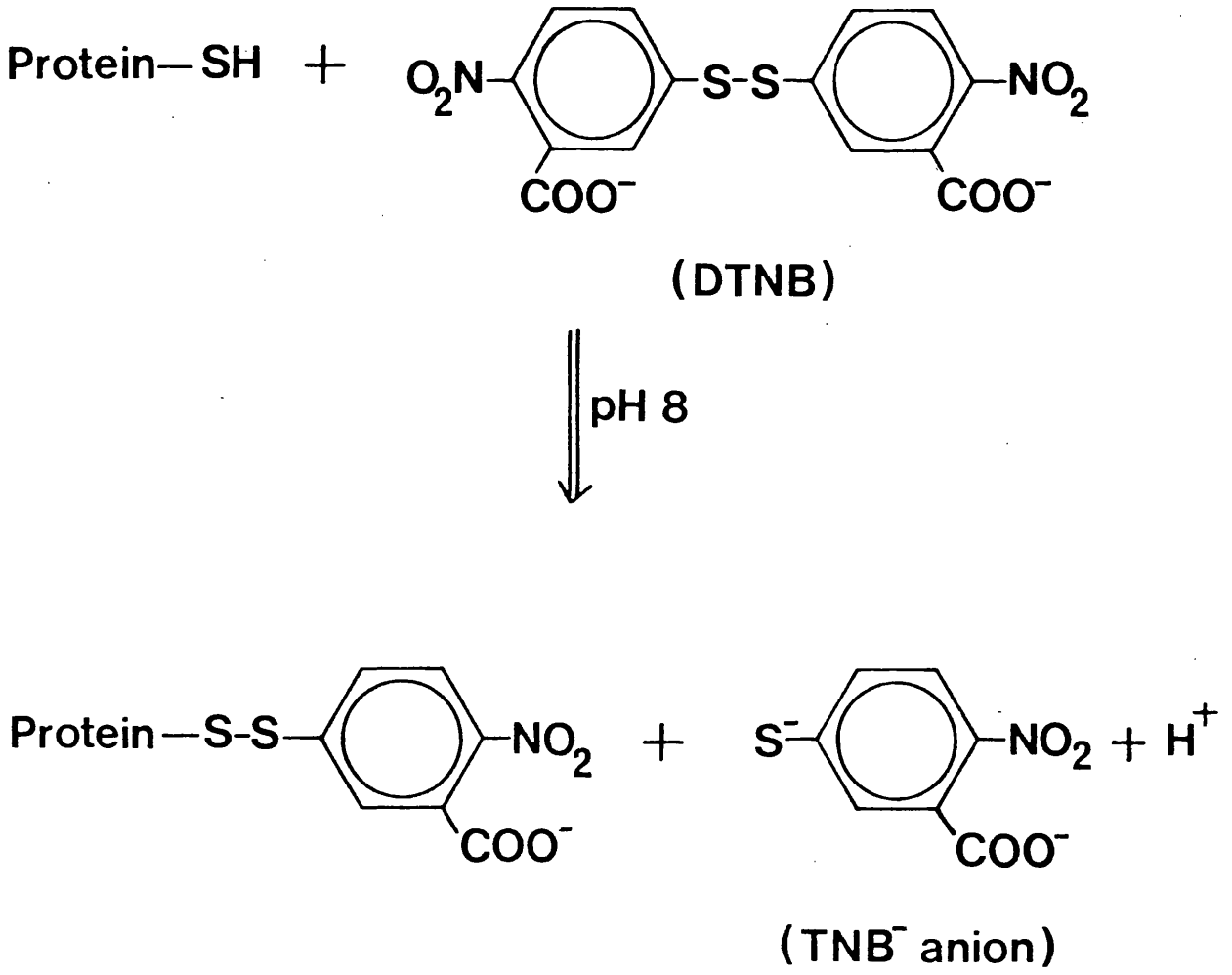


Fig.66. Reaction scheme for the modification of a thiol with DTNB

desensitization to NADH inhibition. Sensitivity to NADH was restored by treatment of the modified enzyme with dithiothreitol which showed that the reaction of DTNB with the enzyme thiol groups was reversible. These results implied that there are two sets of thiol groups in this enzyme, one of which is involved in the activity of the enzyme and the other in the response to NADH. CS I was more rapidly inactivated by DTNB than the wild-type enzyme. There was a rapid desensitization to NADH inhibition and a much slower loss of AMP activation. This correlated with the results from the photo-oxidation experiments which indicated separate binding sites for NADH and AMP. CS II was not affected by DTNB.

There are a number of reasons why proteins which contain cysteine residues should respond to thiol-blocking reagents to different extents. The simplest reason in the case of citrate synthase is that the thiol groups may only be involved in the catalytic and regulatory properties of the 'large' enzyme and not of the 'small' enzyme. Treatment of the 'small' enzyme with DTNB will therefore not result in loss of activity.

Srere (1965 a) showed that pig heart citrate synthase contains thiol groups though they do not react readily with DTNB. Treatment of the enzyme with a denaturing

reagent (urea) resulted in reaction with DTNB. He concluded from this that the thiol groups may have been involved in the catalytic activity but were buried in the native enzyme and consequently were 'masked' from the DTNB.

Nicolau and Bacila (1969) showed that rabbit muscle aldolase contained 28 thiol groups though only 4-6 of these groups reacted with the hydrophilic reagent DTNB in the native enzyme. Denaturation with sodium n-palmitoyl sarcosinate caused additional thiol groups to become exposed. They concluded that enzyme activity was dependent upon the more hidden thiol groups and persisted until 14 of the thiol groups had been exposed and reactive to DTNB.

Placement of thiol groups in hydrophobic regions could occur either by folding of a single protein molecule or by the aggregation of subunits. As well as a total burial of the thiol groups in the molecule, lack of reaction may be due to local steric hindrance. The local steric factors can drastically affect the reaction of thiol groups. Danson and Weitzman (1977) showed that assaying DTNB-treated E.coli citrate synthase in the presence of KCl restored the activity of the enzyme. They concluded that the modified thiols have an indirect

role, such as the maintenance of an active conformation, and that the introduction of several bulky thionitrobenzoate groups into the active site region could cause loss of activity through steric hindrance. If this is the case it is quite feasible that DTNB may not be able to react with certain thiol groups due to local steric hindrance. It may be necessary to use smaller molecules and indeed Degani and Patchornik (1971) have introduced the reagent 2-nitro-5-thiocyanobenzoic acid (NTCB) which reacts with thiol groups to form the S-CN derivative. The cyano substituent has the advantage of being small and uncharged and may be a more reliable probe for the true involvement of thiol groups in enzyme behaviour.

Birchmeier et al. (1973) showed that treatment of thiol groups in aspartate aminotransferase with DTNB resulted in almost total loss of enzyme activity. However, after displacement of the large enzyme-bound thionitrobenzoate by the cyano group there was 60% restoration of activity. This showed that the bulky or charged substituents could impair catalytic activity, even though the cysteine residues were not actually involved in the catalytic mechanism.

Grassetti and Murray (1967) prepared 2-PDS and 4-PDS to determine thiol groups in proteins (Fig.67) These compounds react with thiols to give the corresponding

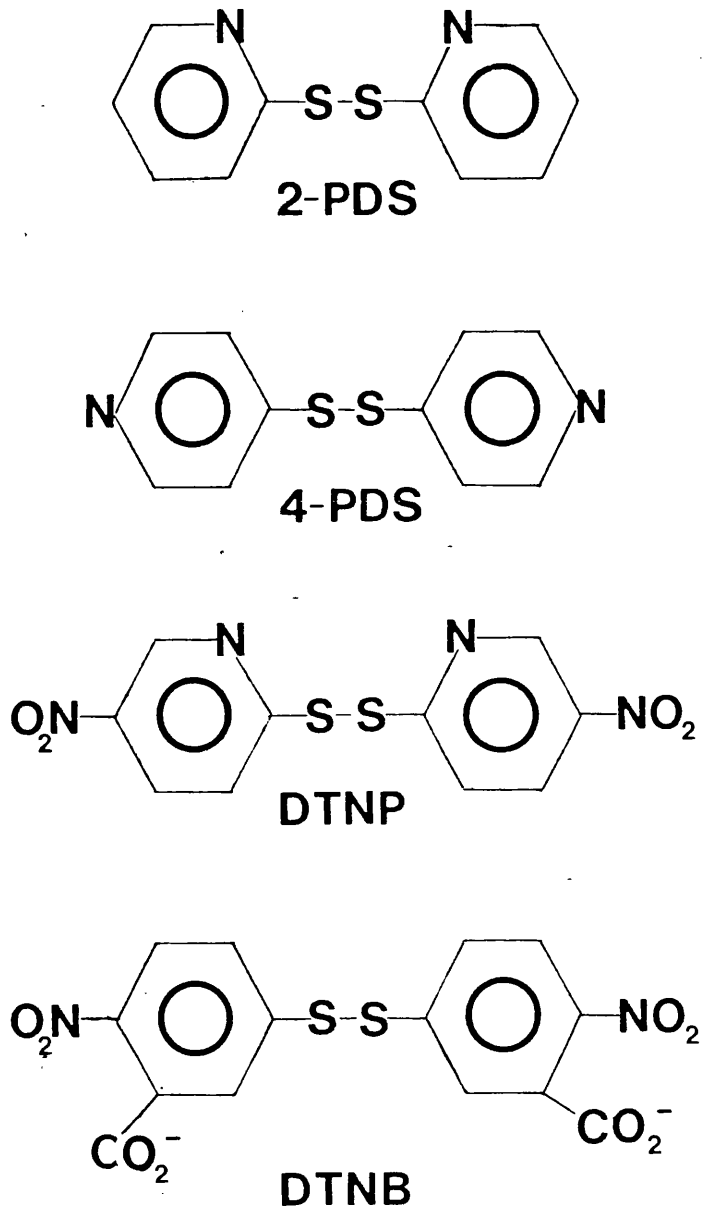


Fig.67. Structure of aromatic disulphides used in this investigation

2- or 4-thiopyridone which absorb at 343 nm and 324 nm respectively. DTNP was also synthesized by Grassetti and Murray (1969) and this compound reacts with thiol groups to release thionitropyridone which absorbs at 386 nm (Fig.67). These compounds were used to study the thiol groups in various citrate synthases.

Zaidenzaig and Shaw (1978) showed that chloramphenicol acetyltransferase is insensitive to inactivation by DTNB and iodoacetate, though the enzyme is sensitive to inactivation by both 2-PDS and 4-PDS. They proposed a hypothesis for the difference in reactivity between these thiol-blocking reagents. The non-reactivity of the carboxylate reagents DTNB and iodoacetate was proposed to be due to electrostatic repulsion by an ionised aspartate or glutamate residue in the vicinity of the chloramphenicol binding site, whereas both 2-PDS and 4-PDS lack carboxyl-groups and can therefore react with the thiol groups.

The electrostatic charge surrounding the cysteine residues influences their reactivity towards thiol-blocking reagents. Talgoy et al. (1979) showed that E.coli citrate synthase was rapidly desensitized to NADH inhibition by DTNB but was inactivated by 4-PDS, and Morse and Duckworth (1980) showed that A.calcoaceticus citrate synthase was

unaffected by DTNB but did react with 4-PDS. These are probably other examples of the electrostatic environment of the thiol groups influencing their reactivity and emphasize how important it is to use more than one type of thiol-blocking reagent when investigating the roles of cysteine residues in enzyme function.

The wild-type Ps.aeruginosa citrate synthase was shown to be far more sensitive to inactivation by 2-PDS and 4-PDS than by DTNB and DTNP. Modification in the presence of salt did not enhance the rate of inactivation. CS I was similarly affected, but none of these reagents inactivated CS II.

The responses of the two 'large' enzymes suggest they may be closely related to one another and that the environment around the cysteine residues may be similar, whereas, in the 'small' enzyme, the thiol groups are either non-essential or are 'masked' as suggested is the case with pig heart citrate synthase (Srere, 1965 a). However, preliminary investigations with 2-PDS and 4-PDS showed a slight inactivation of pig heart citrate synthase, suggesting that cysteine residues may yet be involved in the activity of this enzyme.

The inactivation of A.calcoaceticus citrate synthase by 2-PDS, 4-PDS and DTNP, but not by DTNB, was an extension of the findings of Morse and Duckworth (1980) who also showed that DTNB had no effect on enzyme activity. It is interesting that the inactivation of both E.coli and A.calcoaceticus citrate synthases was enhanced by salt whereas no such effect was observed with any of the Ps.aeruginosa citrate synthases. This may be related to the findings of Morse and Duckworth (1980) that there is greater sequence homology between the citrate synthases of E.coli and A.calcoaceticus than between the citrate synthases of E.coli and Ps.aeruginosa.

The enhancement of the rate of inactivation of certain citrate synthases by thiol-blocking reagents in the presence of salt might be due to an induced conformational change in the enzyme rendering the thiol groups more accessible. Danson and Weitzman (1977) showed that assaying E.coli citrate synthase in the presence of 0.1 M-KCl overcame the inactivation of the enzyme by DTNB, consistent with a conformational change in the enzyme induced by salt. Weitzman and Packman (unpublished observations) also demonstrated that inactivation of the enzyme was greatly enhanced in the presence of 10 mM-$MgCl_2$ which may also have been due to a conformational change.

Warren and Cheatum (1966) showed that neutral salts, at concentrations which only partially inhibited the activity of β-amylase, increased the rate of reaction of its thiol groups with DTNB. They interpreted these observations as providing evidence that salts do indeed alter enzyme activity by altering the structure of the protein macromolecule.

Concluding Remarks

This project was intended to be a continuation of the characterization of a series of mutant E.coli citrate synthases isolated by Harford and Weitzman (1978). However, the finding that a mutant Ps.aeruginosa contained both a 'large' and a 'small' citrate synthase warranted further investigation and complemented studies on other mutant bacterial citrate synthases. Subsequent investigations showed that the ratio of the two enzymes varied according to the growth state of the organism and the nature of the carbon source in the growth medium.

This is the first observation of the occurrence of two growth-phase dependent forms of citrate synthase in the same organism and it provides a unique opportunity to study the structure-function relationships of 'large' and 'small' citrate synthases. The 'large' citrate synthase was found to be sensitive to feedback inhibition by NADH

and activation by AMP, whereas the 'small' citrate synthase was not regulated by these effectors and appeared not to be derived from the 'large' enzyme. The mapping of the wild-type and mutant Ps.aeruginosa citrate synthase genes will reveal whether this is indeed the case.

Investigations of the regulatory properties of CS I have indicated separate NADH and AMP binding sites. This has not previously been seen in aerobic Gram-negative bacteria; the effect of AMP had only been observed in the presence of NADH.

Future success in the purification of the wild-type and the two mutant enzymes will permit a more detailed investigation of the properties of, and structural similarities and differences between, the three Pseudomonas enzymes. Such comparative studies should further our general understanding of the molecular basis of the cellular function of citrate synthase.

APPENDIX

The two programs listed below were originally obtained from Professor W.W. Cleland, Department of Biochemistry, University of Wisonsin.

Program I - was used when reciprocal plots of initial velocity vs. substrate concentration were linear; the apparent Km value was computed.

Program II - was used in the calculation of the $S_{0.5}$ value for a substrate in cases where reciprocal plots of initial velocity vs. substrate concentration were non-linear.

PROGRAM I

```
 dimension v(100), a(100), w(100), s(3,4),q(3), sm(3),ss(3)
&,y(100),recv1(100),recv2(100),reca(100),sv1(100),sv2(100)
 write (6,100)
 100 format(35h fit to hyperbola v=vmax*a/(k+a)   ///)
11 format(v)
1 format(v)
 500 format(5h one          )
 300 format(//      )
 jj = 0
 14 read (5,11) np
 if ( np ) 99,99,12
 12 m = 1
 n = 2
 p = np-n
 n1 = n+1
 n2 = n+2
 go to 2
 15 read (5,1) v(i), a(i), w(i)
 if (w(i)) 19,19,20
  19 w(i) = 1.0
 20 q(1) = v(i)**2/a(i)
  q(2) =v(i)**2
 q(3) = v(i)
 go to 13
 16 ck = s(1,1)/ s(2,1)
 jj = jj+1
 write (6,300)
 write (6,11)  jj
 nt = 0
 m = 2
 go to 2
 17  d = ck + a(i)
 q(1) =a(i) /d
 q(2) =q(1) /d
 q(3) = v(i)
 go to 13
 18 ck = ck -s(2,1) / s(1,1)
 nt = nt + 1
 if (nt-5) 2,21,21
 21 s2 = 0
 do 22 i=1,np
 22 s2 = s2 + (v(i) - s(1,1)*a(i) / (ck+a(i) ))**2 * w(i)
 s2 = s2 / p
 s1 = sqrt (s2)
 vint = 1./s(1,1)
 sl =ck / s(1,1)
 vk =1./sl
 do 10 j = 2,n1
 do 10 k =1,n
 10 s(k,j) = s(k,j) * sm(k) * sm(j-1)
 sev = s1 * sqrt ( s(1,2) )
  seck=s1 * sqrt ( s(2,3) ) / s(1,1)
 sevi = sev/s(1,1)**2
 s(1,3) = s1*sqrt (ck**2*s(1,2) + s(2,3) + 2.*ck*s(1,3) )
 sesl = s(1,3) / s(1,1)**2
 sevk = s(1,3) / ck**2
 wck = 1./seck**2
 wv = 1./ sev**2
 wvint =1./ sevi**2
 wvk =1./ sevk**2
 wsl = 1./ sesl**2
```

continued

PROGRAM I (continued)

```
write (6,30) ck, seck,wck
write (6,31) s(1,1),sev,wv
write (6,32) sl,sesl,wsl
write (6,33) vint, sevi , wvint
write (6,34) vk,sevk,wvk
write (6,35) s2, s1
30 format (7h k    = ,f12.6,13h s.e.(k)    = ,f11.6,5h w = ,e14.5 )
31 format (7h v  = ,f12.6,13h s.e. (v)    = ,f11.6,5h w = ,e14.5 )
32 format  (7h k/v = ,f12.6,13h s.e. (k/v) = ,f11.6,5h w = ,e14.5 )
33 format (7h 1/v = ,f12.6,13h s.e. (1/v) = ,f11.6,5h w = ,e14.5 )
34 format (7h v/k = ,f12.6,13h s.e.(v/k) = ,f11.6,5h w= ,e14.5 )
35 format  (12h variance = ,e14.5,10h sigma = ,f12.7// )
write(6,104)
104 format ( 100h     v          s        best v     1/v         1/s      be
 &st 1/v    s/v      best s/v  weight                                )
do 50 i=1,np
y(i) = s(1,1)*a(i) / (a(i)+ck)
recv1(i) = 1./ v(i)
recv2(i) = 1. / y(i)
reca(i)= 1. /a(i)
sv1(i) = a(i) /v(i)
sv2(i) = a(i) /y(i)
105 format (9f10.5)
 50 write (6,105)v(i),a(i),y(i),recv1(i),reca(i),recv2(i),sv1(i),sv2(i)
&,w(i)
go to 14
2 do 3 j=1,n2
do 3 k =1,n1
3 s(k,j) = 0
do 4 i=1,np
 go to (15,17) , m
13 do  4 j = 1,n1
do 4 k =1,n
4 s(k,j) =s(k,j) + q(k)*q(j)*w(i)
do 5  k = 1,n
5 sm(k) = 1. / sqrt (s(k,k) )
sm(n1) = 1.0
do 6 j =1,n1
do 6 k =1,n
6 s(k,j) = s(k,j) * sm(k) * sm(j)
ss(n1) = -1.0
s(1,n2) = 1.0
do 8 i =1,n
do 7 k = 1,n
7 ss(k) = s(k,1)
do  8 j = 1,n1
do 8 k = 1,n
8 s(k,j) = s(k+1,j+1) - ss(k+1) * s(1,j+1) / ss(1)
do  9 k =1,n
9 s(k,1)=s(k,1) * sm(k)
go to (16,18), m
36 format (23h program completed  for ,i4, 6h lines  )
99 write (6,36)  jj
stop
end
```

PROGRAM II

```
dimension v(100),a(100),s(4,5),q(4,100),sm(4),ss(4)
dimension recv1(100),recv2(100),reca(100),y(100),hill1(100),hill2(100),hill3(100)
 write(6,100)                                                    slope(100)
 100 format(32h fit to y = v*x**2/(a+2b*x+x**2)      ///)
11 format(i3,65h
 1 format(v)
 105 format( ///)
 jj = 0
 14 read(5,11)np
 if (np) 99,99,12
 12 m = 1
 n = 3
 p = np-n
 n1= n+1
 n2= n+2
 write(6,105)
 go to 2
 15 read(5,1) v(i), a(i)
 q(1,i) = v(i)**2/a(i)**2
 q(2,i) = v(i)**2/a(i)
 q(3,i) = v(i)**2
 q(4,i) = v(i)
 go to 13
 16 ca = s(1,1) / s(3,1)
 cb = .5*s(2,1) /s(3,1)
 jj = jj+1
 write(6,11) jj,np
 nt = 0
 m = 2
 go to 2
 17 d = ca + 2.*cb*a(i) + a(i)**2
 q(1,i) = a(i)**2/d
 q(2,i) = a(i)**2/d**2
 q(3,i) = a(i)**3/d**2
 q(4,i) = v(i)
 go to 13
 18 ca = ca - s(2,1)/s(1,1)
 cb = cb - s(3,1)/(2.*s(1,1))
 write(6,102) ca, cb, s(1,1)
 nt = nt+1
 if(nt-6) 2, 87, 87
 87 s2 = 0
 cv2= s(1,1)
 c1v= 1./s(1,1)
do 82 i=1,np
 82 s2 = s2+(v(i)-cv2*a(i)**2/(ca+2.*cb*a(i)+a(i)**2))**2
 s2 = s2/p
 s1 = sqrt(s2)
 do 10 j=2,4
 do 10 k=1,3
 10 s(k,j) = s(k,j)*sm(k)*sm(j-1)
 sea = s1*sqrt(s(2,3))/ s(1,1)
 seb = .5*s1*sqrt(s(3,4)) /s(1,1)
 sev = s1*sqrt(s(1,2))
 se1v = sev/s(1,1)**2
 wv = 1./sev**2
 w1v = 1./se1v**2
 write(6,37) ca, sea
 write(6,38) cb, seb
 write(6,39) cv2, sev, wv
 write(6,40) c1v, se1v, w1v
```

continued

PROGRAM II (continued)

```
37 format(6h  a = ,f12.6,15h   s.e.(a)   = , f11.6)
38 format(6h  b = ,f12.6,15h   s.e.(b)   = ,F11.6)
40 format(6h 1/v= ,f12.6,15h   s.e.(1/v) = , f11.6,6h  w = ,e14.5 )
39 format(6h  v = ,f12.6,15h   s.e.(v)   = , f11.6,6h  w = ,e14.5 )
write(6,44) s2
44 format(12h variance =, e14.5 ///)
write(6,101)
101 format(110h    s          v       best v     1/s        1/v   best
&1/v     lg s     lg v/vm-v  best v/vm-v  slope                 /)
do 50 i=1,np
y(i) =cv2*a(i)**2 / (ca+2*cb*a(i)+a(i)**2)
reca(i)  = 1. / a(i)
recv1(i) = 1. / v(i)
recv2(i) = 1. / y(i)
hill1(i) = alog10(v(i)/(cv2-v(i) ) )
hill2(i) = alog10(y(i)/(cv2-y(i) ) )
hill3(i) = alog10(a(i))
slope(i) = 2.*ca/cv2/a(i) +2.*cb/cv2
102 format(10f10.5)
50 write(6,102) a(i),v(i),y(i),reca(i),recv1(i),recv2(i),hill3(i),
& hill1(i), hill2(i), slope(i)
go to 14
2 do 3 j=1,n2
do 3 k=1,n1
3 s(k,j) = 0
do 4 i=1,np
go to (15,17), m
13 do 4 j =1,n1
do 4 k =1,n
4 s(k,j) = s(k,j) +q(k,i)*q(j,i)
do 5 k=1,n
5 sm(k) = 1. / sqrt (s(k,k) )
sm(n1) = 1.0
do 6 j = 1,n1
do 6 k = 1,n
6 s(k,j) = s(k,j) * sm(k) * sm(j)
ss(n1) = -1.0
s(1,n2) = 1.0
do 8 i = 1,n
do 7 k = 1,n
 7 ss(k) = s(k,1)
do 8 j = 1,n1
do 8 k = 1,n
8 s(k,j) = s(k+1,j+1) - ss(k+1) * s(1,j+1) / ss(1)
do 9 k =1,n
9 s(k,1) =s(k,1) * sm(k)
go to (16,18), m
 36 format (23h program completed for ,i4, 6h lines  )
99 write  6,36)  jj
stop
end
```

BIBLIOGRAPHY

"Affinity Chromatography, Principles and Methods" (1974), pp.31-34, Pharmacia Fine Chemicals, Box 175, 5-751 04 Uppsala 1, Sweden

Amarasingham, C.R. and Davis, B.D. (1965) J.Biol.Chem. 240, 3664-3668

Andrews, P. (1965) Biochem.J. 96, 595-606

Ashworth, J.M. and Kornberg, H.L. (1966) Proc.Roy.Soc.(London) B,165, 179-188

Ashworth, J.M., Kornberg, H.L. and Nothmann, D.L. (1965) J.Mol.Biol. 11, 654-657

Atkinson, D.E. (1968) In "Metabolic Roles of Citrate" (Goodwin, T.W., ed.), pp.23-40, Academic Press, New York

Axen, R., Porath, J. and Ernback, S. (1967) Nature (London) 214, 1302-1304

Ball, D.J. and Nishimura, J.S. (1980) J.Biol.Chem. 255, 10805-10812

Barry, S. and O'Carra, P. (1973) FEBS Lett. 37, 134-139

Barry, S., Brodelius, P. and Mosbach, K. (1976) FEBS Lett. 70, 261-266

Bayer, E., Bauer, B. and Eggerer, H. (1981 a) FEBS Lett. 123, 258-260

Bayer, E., Bauer, B. and Eggerer, H. (1981 b)
FEBS Lett. 127, 101-104

Bethell, M.R. and Jones, M.E. (1969)
Arch.Biochem.Biophys. 134, 352-365

Birchmeier, W., Wilson, K.J. and Christen, P. (1973)
J.Biol.Chem. 248, 1751-1759

Bogin, E. and Wallace, A. (1966)
Biochim.Biophys.Acta 128, 190-192

Brocklehurst, K. (1979)
Int.J.Biochem. 10, 259-274

Caggiano, A.V. and Powell, G.L. (1979)
J.Biol.Chem. 254, 2800-2806

Canovas, J.L. and Kornberg, H.L. (1966)
Proc.Roy.Soc.(London) B,165, 189-205

Cazzulo, J.J. (1973)
FEBS Lett. 30, 339-342

Chapman, A.G., Fall, L. and Atkinson, D.E. (1971)
J.Bacteriol. 108, 1072-1086

Chibata, I., Tosa, T. and Matua, Y. (1974)
Methods Enzymol. 34, 267-271

Chrambach, A., Reisfeld, R.A., Wyckoff, M. and Zaccari, J. (1967)
Anal.Biochem. 20, 150-154

Citri, N. (1973)
Adv.Enzymol. 37, 397-648

Clarke, P.H. and Meadow, P.M. (1959)
J.Gen.Microbiol. 20, 144-155

Coleman, M.S. and Jones, M.E. (1971)
Biochemistry 10, 3390-3396

Cornish-Bowden, A. (1978)
J.Theor.Biol. 74, 155-161

Cuatrecassas, P. (1970)
J.Biol.Chem. 245, 3059-3065

Danson, M.J. and Weitzman, P.D.J. (1973)
Biochem.J. 135, 513-524

Danson, M.J. and Weitzman, P.D.J. (1977)
Biochim.Biophys.Acta 485, 452-464

Danson, M.J., Harford, S. and Weitzman, P.D.J. (1979)
Eur.J.Biochem. 101, 515-521

Davis, B.D. (1961)
Cold Spring Harbor Symp.Quant.Biol. 26, 1-10

Davis, B.J. (1964)
Ann.N.Y.Acad.Sci. 121, 404-427

Dean, P.D.G. and Lowe, C.R. (1972)
Biochem.J. 127, 11P-12P

Degani, Y. and Patchornik, A. (1971)
J.Org.Chem. 36, 2727-2728

Deshpande, W.M. and Ramakrishnan, C.V. (1959)
J.Biol.Chem. 234, 1929-1930

Dixon, M. and Webb, E.C. (1964)
In "Enzymes", p.551, Longman Group Ltd., London

Eidels, L. and Preiss, J. (1970)
J.Biol.Chem. 245, 2937-2945

Ellman, G.L. (1959)
Arch.Biochem.Biophys. 82, 70-77

Flechtner, V.R. and Hanson, R.S. (1969)
Biochim.Biophys.Acta 184, 252-262

Flechtner, V.R. and Hanson, R.S. (1970)
Biochim.Biophys.Acta 222, 253-264

Gilham, P.T. (1971)
Methods Enzymol. 21, 191-197

Goodman, M.F. and Bessman, M.J. (1973)
J.Biol.Chem. 248, 815-821

Gorini, L. and Maas, W.K. (1957)
Biochim.Biophys.Acta 25, 208-209

Grassetti, D.R. and Murray, J.F. (1967)
Arch.Biochem.Biophys. 119, 41-49

Grassetti, D.R. and Murray, J.F. (1969)
J.Chromat. 41, 121-123

Gray, C.T., Wimpenny, J.W.T. and Mossman, M.R. (1966)
Biochim.Biophys.Acta 117, 33-41

Harford, S. (1977)
Ph.D. Thesis, University of Leicester

Harford, S. and Weitzman, P.D.J. (1975)
Biochem.J. 151, 455-458

Harford, S. and Weitzman, P.D.J. (1978)
Biochem.Soc.Trans. 6, 433-435

Harmey, M.A. and Neupert, W. (1979)
FEBS Lett. 108, 385-389

Hathaway, J.A. and Atkinson, D.E. (1965)
Biochem.Biophys.Res.Commun. 20, 661-665

Henderson, C.E., Perham, R.N. and Finch, J.T. (1979)
Cell, 17, 85-93

Heron, E.J. and Caprioli, R.M. (1975)
Biochim.Biophys.Acta 403, 563-571

Higa, A.I., Massarini, E. and Cazzulo, J.J. (1978)
Can.J.Microbiol. 24, 215-221

Hillier, S. and Charnetzky, W.T. (1981)
J.Bacteriol. 145, 452-458

Hogness, D.S., Cohn, M. and Monod, J. (1955)
Biochim.Biophys.Acta 16, 99-116

Hy, M. and Reeves, H.C. (1976)
Biochim.Biophys.Acta 445, 280-285

Johnson, D.E. and Hanson, R.S. (1974)
Biochim.Biophys.Acta 350, 336-353

Jones, D. and Weitzman, P.D.J. (1971)
J.Gen.Microbiol. 69, xi

Jones, D. and Weitzman, P.D.J. (1974)
Int.J.Syst.Bacteriol. 24, 113-117

Kantrowitz, E.R., Foote, J., Reed, H.W. and Vensel, L.A. (1980)
Proc.Natl.Acad.Sci.USA 77, 3249-3253

Koch, A.I. and Levy, H.R. (1955)
J.Biol.Chem. 217, 947-957

Kogut, M. and Podoski, E.P. (1953)
Biochem.J. 55, 800-811

Kornberg, A. (1955 a)
Methods Enzymol. 1, 441-443

Kornberg, A. (1955 b)
Methods Enzymol. 1, 705-707

Kornberg, H.L. (1959)
Ann.Rev.Microbiol. 13, 49-78

Kornberg, H.L. (1966)
Biochem.J. 99, 1-11

Kornberg, H.L. and Krebs, H.A. (1957)
Nature (London) 179, 988-991

Krebs, H.A. and Johnson, W.A. (1937)
Enzymologia 4, 148-156

Krebs, H.A. and Lowenstein, J.M. (1960)
In "Metabolic Pathways" (2nd Edition. Greenberg, D.M., ed.), 1, 129-203, Academic Press, New York

Krebs, H.A., Gurin, S. and Eggleton, L.V. (1952)
Biochem.J. 51, 614-627

Lamed, R., Levin, Y. and Wilchek, M. (1973)
Biochim.Biophys.Acta 304, 231-235

Lanoue, K.F., Bryla, J. and Williamson, J.R. (1972)
J.Biol.Chem. 247, 667-679

Larrabee, K.L., Phillips, J.O., Williams, G.J. and Larrabee, A.R. (1980)
J.Biol.Chem. 255, 4125-4130

Layne, E. (1957)
Methods Enzymol. 3, 447-454

Lee, L.P.K. and Kosicki, G.W. (1967)
Biochim.Biophys.Acta 139, 195-198

Lessie, T.G. and Neidhart, F.C. (1980)
J.Bacteriol. 93, 1800-1810

Lowe, C.R. and Dean, P.D.G. (1974)
In "Affinity Chromatography", pp.92-109,
John Wiley and Sons Ltd., London, U.K.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and
Randall, R.J. (1951)
J.Biol.Chem. 193, 265-275

Lucas, C. and Weitzman, P.D.J. (1977)
Arch.Microbiol. 114, 55-60

Lynen, F. and Reichert, E. (1951)
Angew.Chem. 63, 47-48

Macnab, R., Moses, V. and Mowbray, J. (1973)
Eur.J.Biochem. 34, 15-19

Magasanik, B. (1961)
Cold Spring Harbor Symp.Quant.Biol. 26, 249-256

Massarini, E. and Cazzulo, J.J. (1974)
FEBS Lett. 39, 252-254

Massarini, E. and Cazzulo, J.J. (1975)
FEBS Lett. 57, 134-138

Matlib, M.A., Boesman-Finkelstein, M. and Srere, P.A. (1978)
Arch.Biochem.Biophys. 191, 426-430

Morse, D. and Duckworth, H.W. (1980)
Can.J.Biochem. 58, 696-706

Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. (1972)
Biochem.J. 127, 625-631

Mukherjee, A. and Srere, P.A. (1976)
J.Biol.Chem. 251, 1476-1480

Murphey, W.H., Kitto, G.B., Everse, J. and Kaplan, N.O. (1967)
Biochemistry 6, 603-609

Nicolau, J. and Bacila, M. (1969)
Arch.Biochem.Biophys. 129, 357-361

Nierhaus, D. and Nierhaus, K.H. (1973)
Proc.Natl.Acad.Sci.USA 70, 2224-2228

O'Carra, P., Barry, S. and Griffin, T. (1973)
Biochem.Soc.Trans. 1, 289-290

Ochoa, S. (1955)
Methods Enzymol. 1, 735-739

Ochoa, S., Stern, J.R. and Schneider, M.C. (1951)
J.Biol.Chem. 193, 691-702

Parker, M.G. and Weitzman, P.D.J. (1970)
FEBS Lett. 7, 324-326

Passonneau, J.V. and Lowry, O.H. (1962)
Biochem.Biophys.Res.Commun. 7, 10-15

Polakis, E.S. and Bartley, W. (1965)
Biochem.J. 97, 284-297

Ramakrishnan, C.V. and Martin, S.N. (1954)
Can.J.Biochem.and Physiol. 32, 434-439

Roberts, R.B., Cowie, D.B., Britten, R., Bolton, E. and Abelson, P.H. (1953)
Proc.Natl.Acad.Sci.USA 39, 1013-1026

Rogers, H.J. and Perkins, H.R. (1968)
In "Cell Walls and Membranes" (Long, C., ed.), pp.206-230, E. and F.N. Spon Ltd., London

Rowe, A.J. and Weitzman, P.D.J. (1969)
J.Mol.Biol. 43, 345-349

Salton, M.R.J. (1964)
In "The Bacterial Cell Wall", pp.32-36
Elsevier Publishing Co., Amsterdam

Sanwal, B.D. (1970)
Bacteriol.Rev. 34, 20-39

Scamuffa, M.D. and Caprioli, R.M. (1980)
Biochim.Biophys.Acta 614, 583-590

Self, C.H. and Weitzman, P.D.J. (1972)
Biochem.J. 130, 211-219

Singh, M., Brooks, G.C. and Srere, P.A. (1970)
J.Biol.Chem. 245, 4636-4640

Skinner, A.J. and Clarke, P.H. (1968)
J.Gen.Microbiol. 50, 183-194

Spector, L.B. (1972)
In "The Enzymes" (3rd Edition. Boyer, P.D., ed.), 7, 357-389, Academic Press, New York

Srere, P.A. (1965 a)
Biochem.Biophys.Res.Commun. 18, 87-91

Srere, P.A. (1965 b)
Biochim.Biophys.Acta 106, 445-455

Srere, P.A. (1968)
In "Metabolic Roles of Citrate" (Goodwin, T.W., ed.), pp.11-21, Academic Press, New York

Srere, P.A. (1971)
Adv.Enz.Regul. 9, 221-233

Srere, P.A. (1972)
Curr.Top.Cell.Regul. 5, 229-283

Srere, P.A. and Mosbach, K. (1974)
Ann.Rev.Microbiol. 28, 61-83

Srere, P.A., Brazil, H. and Gonen, L. (1963)
Acta Chem.Scand. 17, S129-S134

Stadtman, E.R. (1957)
Methods Enzymol. 3, 931-932

Stanier, R.T., Palleroni, N.J., Doudoroff, M. (1966)
J.Gen.Microbiol. 43, 159-271

Stern, J.R. and Ochoa, S. (1949)
J.Biol.Chem. 179, 491-492

Stern, J.R. and Ochoa, S. (1951)
J.Biol.Chem. 191, 161-172

Stern, J.R., Shapiro, B. and Ochoa, S. (1950)
Nature (London) 166, 403-404

Swissa, M. and Benziman, M. (1976)
Biochem.J. 153, 173-179

Talgoy, M.M., Bell, A.W. and Duckworth, H.W. (1979)
Can.J.Biochem. 57, 822-833

Taylor, A.L. and Trotter, C.D. (1972)
Bacteriol.Rev. 36, 504-524

Tong, E.K. and Duckworth, H.W. (1975)
Biochemistry 14, 235-241

Umbarger, H.E. (1954)
J.Bacteriol. 68, 140-145

Umbarger, H.E. (1956)
Science 123, 848

Wallenfels, K. (1962)
Methods Enzymol. 5, 212-219

Warren, J.C. and Cheatum, S.G. (1966)
Biochemistry 5, 1702-1707

Weil, L. (1965)
Arch.Biochem.Biophys. 110, 57-68

Weil, L. and Maher, J. (1950)
Fed.Proc.Fed.Amer.Soc.Exp.Biol. 9, 244

Weil, L., Gordon, W.G. and Buchert, A.R. (1951)
Arch.Biochem.Biophys. 33, 90-109

Weitzman, P.D.J. (1966 a)
Biochem.J. 101, 44c-45c

Weitzman, P.D.J. (1966 b)
Biochim.Biophys.Acta 128, 213-215

Weitzman, P.D.J. (1969 a)
Methods Enzymol. 13, 22-25

Weitzman, P.D.J. (1969 b)
Methods Enzymol. 13, 365-368

Weitzman, P.D.J. (1973)
FEBS Lett. 32, 247-250

Weitzman, P.D.J. (1978)
J.Gen.Microbiol. 106, 383-386

Weitzman, P.D.J. (1981)
Adv.Microb.Physiol. 22, 185-244

Weitzman, P.D.J. and Danson, M.J. (1976)
Curr.Top.Cell.Regul. 10, 161-204

Weitzman, P.D.J. and Dunmore, P. (1969 a)
Biochim.Biophys.Acta 171, 198-200

Weitzman, P.D.J. and Dunmore, P. (1969 b)
FEBS Lett. 3, 265-267

Weitzman, P.D.J. and Hewson, J.K. (1973)
FEBS Lett. 36, 227-231

Weitzman, P.D.J. and Jones, D. (1968)
Nature (London) 219, 270-272

Weitzman, P.D.J. and Kinghorn, H.A. (1978)
FEBS Lett. 88, 255-288

Weitzman, P.D.J., Ward, B.A. and Rann, D.L. (1974)
FEBS Lett. 43, 97-100

Weitzman, P.D.J., Kinghorn, H.A., Beecroft, L.J. and Harford, S. (1978)
Biochem.Soc.Trans. 6, 436-438

Wiebe, W.J. and Bancroft, K. (1975)
Proc.Natl.Acad.Sci.USA 72, 2112-2115

Wieland, O. and Weiss, L. (1963)
Biochem.Biophys.Res.Commun. 13, 26-31

Wimpenny, J.W.T. (1979)
J.Gen.Microbiol. 114, 483-486

Wimpenny, J.W.T. and Parr, J.A. (1979)
J.Gen.Microbiol. 114, 487-489

Wright, J.A. and Sanwal, B.D. (1971)
J.Biol.Chem. 246, 1689-1699

Wright, J.A., Maeba, P. and Sanwal, B.D. (1967)
Biochem.Biophys.Res.Commun. 29, 34-38

Yates, R.A. and Pardee, A.B. (1956)
J.Biol.Chem. 221, 757-770

Zaidenzaig, Y. and Shaw, W.V. (1978)
Eur.J.Biochem. 83, 553-562